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ERRATUM

In the paper *The effect of magnesium, potassium, and iron on the growth and morphology of red halophilic bacteria*, by Helen J. Brown and N. E. Gibbons, Can. J. Microbiology, 1 : 486-494. 1955, Figure 15 appears over the caption for Figure 1, and vice versa. The error has been corrected in the reprints and the following captions may be substituted for those appearing in the paper.

FIG. 1. Effect of concentration of magnesium on growth of *P. salinaria*, *P. cutirubra*, *H. halobium*, and *S. littoralis*. Curves marked *S* are shake cultures, all others standing cultures.

FIG. 15. Effect of magnesium concentration on growth of *P. salinaria*. Open circles—parent strain, closed circles—strain adapted to low concentration of magnesium.

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NOUVELLE TECHNIQUE POUR L'ÉTUDE *IN VIVO* DE LA SENSIBILITÉ DES STAPHYLOCOQUES AUX SUBSTANCES ANTIBACTÉRIENNES

UTILISATION DE L'EMBRYON DE POULET DE 19 JOURS[1]

PAR S. SONEA ET A. FRAPPIER

Abstract

The method of experimental staphylococcal infection using the 19-day-old chick embryo was adapted to the testing of antibacterial substances. This *in vivo* test consists in administering the antibacterial substance in the air sac of the egg, and in injecting the staphylococci into the embryo itself through the air sac. Contrary to other *in vivo* methods, lethal infections are easily obtained with most of the strains by injecting a very small number of staphylococci into untreated embryos; LD_{50} and MLD determinations are obtained at will with all *Staphylococcus* strains; in this experimental infection, initial toxemia is avoided because the effective inoculum is small and the red cells of the chick embryo are resistant to staphylococcal alpha-toxin. This method is an improvement over others previously described, which used rabbits or mice. The first phases of this infection could be compared in some respects with those of clinical cases. In the case of the *in vitro* sensitive strains, penicillin (50 units per egg) checked the infection when given at any time from the moment of inoculation up to six hours later. Two strains were penicillin resistant *in vitro* and *in vivo* but two other strains were resistant *in vitro* and susceptible *in vivo* to penicillin. Two different sulphonamides were effective against the chick-embryo infections obtained with most of the *Staphylococcus* strains used in this work.

Introduction

Le traitement des infections à staphylocoques affronte des difficultés croissantes. Celles-ci sont dues en grande partie à la fréquence des souches résistantes aux agents thérapeutiques courants, les antibiotiques en particulier.

La détermination *in vitro* de la sensibilité des souches aux divers antibiotiques donne à la clinique des renseignements utiles, mais il y a parfois des contradictions entre les résultats thérapeutiques et les essais de sensibilité. On pourrait probablement éviter une partie de ces difficultés si l'on disposait d'une épreuve *in vivo* fidèle et susceptible de laisser prévoir l'action des

[1] *Manuscrit reçu le 5 mai 1955.*

Contribution du Département de Bactériologie de la Faculté de médecine et de l'Institut de Microbiologie et d'Hygiène de l'Université de Montréal, Montréal, Québec.

Travail effectué à l'aide d'un octroi des Fondations Joseph Rhéaume. Une partie de ce travail a constitué le sujet d'une communication présentée au Congrès de la Can. Public Health Assoc., section laboratoire, à Montréal, le 14 décembre 1954.

[The August number of this Journal (Can J. Microbiol. 1 : 473-595. 1955) was issued August 23, 1955.]

substances antibactériennes chez l'homme. Cette épreuve permettrait éventuellement l'étude et la mise au point de traitements nouveaux des infections à staphylocoques.

Les épreuves de sensibilité aux antibiotiques *in vivo* pratiquées chez la souris inoculée par voie intrapéritonéale (8), sous-cutanée (5), ou intracérébrale (6) ne permettent pas de rapporter les résultats à l'homme. En effet, contrairement à l'homme, la souris présente une sensibilité exceptionnelle aux toxines staphylococciques et d'autre part une plus grande résistance à l'envahissement par les staphylocoques. On doit utiliser dans ces épreuves, des inoculums considérables et l'infection obtenue d'emblée brûle les étapes. Il est alors difficile d'étudier l'action thérapeutique sur les phases précoces de l'envahissement.

Si on veut diminuer le nombre des bactéries dans l'inoculum en utilisant une substance adjuvante (3), on introduit alors des facteurs artificiels qui éloignent davantage l'infection expérimentale des conditions cliniques.

La méthode d'inoculer le jaune d'œuf avec les bactéries (4) peut difficilement être appelée une méthode *in vivo*.

Certains résultats intéressants ont été obtenus en inoculant par voie intraveineuse des poulets d'environ deux livres avec des staphylocoques (7) mais les doses sont encore assez fortes (400 millions de bactéries vivantes) et la méthode présente de sérieuses difficultés d'ordre pratique. Avec les méthodes mentionnées, il est impossible d'obtenir à volonté des infections expérimentales de gravité variée et surtout de déterminer la dose LD_{50} ou la dose minima mortelle.

Nous avons eu l'occasion de mettre au point une méthode d'infection staphylococcique expérimentale, chez l'embryon de poulet âgé de 18 ou 19 jours (1 et 2), au moyen de laquelle les inconvénients cités plus haut sont atténués. Dans le présent travail, nous avons modifié cette méthode pour l'adapter aux essais *in vivo* des substances antistaphylococciques.

Matériel et Méthodes

Des embryons de poulet de 19 jours sont mirés pour en choisir seulement les vivants. On les place, le bout pointu en bas, sur des portoirs en carton. On applique de la teinture d'iode sur toute la surface de l'extrémité opposée de façon à rendre aseptique la coquille qui recouvre la chambre à air.

On pratique par la suite aseptiquement une ouverture d'environ 15 mm. de diamètre dans la coquille qui recouvre la chambre à air. La membrane coquillière est dédoublée à cet endroit et sa partie interne est gardée intacte. A travers cette partie intacte de la membrane coquillière, on voit d'habitude un soulèvement causé par l'embryon. Ce dernier, à ce stade du développement, remplit presque complètement le contenu de l'œuf.

On injecte l'embryon à travers le dédoublement de la membrane coquillière. La fenêtre pratiquée dans la chambre à air est refermée avec une bande de cellophane collant (scotch tape) qu'on peut enlever ou entr'ouvrir facilement

pour appliquer les substances à étudier sur la surface de la membrane coquillière restée intacte. Les œufs inoculés sont déposés à l'étuve à 37° C. pour trois à quatre jours et la lecture des résultats se fait le plus facilement en comptant le nombre de poussins vivants qui éclosent par rapport à celui des embryons morts. On peut aussi ouvrir les œufs de 24 à 48 h. après l'inoculation si on veut obtenir des résultats rapides.

L'*inoculum* contient, sous un volume de 0.1 cc., des staphylocoques en suspension dans l'eau salée physiologique. Ces germes proviennent d'une culture de 24 h. sur gélose.

On peut facilement déterminer au préalable la dose L_{50} ou la dose minima mortelle (tuant 100% des embryons) pour chaque souche de staphylocoques. Nous avons recherché d'abord ces doses pour chaque souche, par des essais effectués au moyen de dilutions variées contrôlées par la numération des colonies. Au moment des expériences thérapeutiques, nous obtenions des doses L_{50} ou minimum mortelles connues en ajustant la suspension bactérienne au moyen de comparaisons turbidimétriques, sans omettre la confirmation ultérieure par comptage des colonies. Nous avons obtenu pour la majorité des souches étudiées une mortalité de 50% ou plus, avec environ cent bactéries vivantes. A toutes fins pratiques et pour obtenir une réponse rapide, il semble que cette quantité de bactéries puisse être utilisée d'emblée avec la plupart des souches inconnues. Quelques rares souches ont donné le même résultat avec 10 bactéries ou moins. Nous avons rencontré quelques souches peu virulentes pour les embryons de poulet, qui ont nécessité un inoculum d'environ 100,000 bactéries vivantes ou plus pour produire une mortalité suffisante. Pour une souche donnée, la quantité de staphylocoques nécessaire pour obtenir la dose LD_{50} et la dose minima mortelle est constante d'une expérience à l'autre.

Pour des tests cliniques *in vivo*, avec des souches inconnues on peut utiliser comme dose infectante une dilution au dix-millième ou au cent-millième d'une culture de 24 h. sur bouillon ordinaire. Dans la grande majorité des cas, la mortalité obtenue dans ces conditions se situe entre 50% et 100%.

Toutes les souches de staphylocoques utilisées dans cette étude coagulaient le plasma de lapin. Les souches A8 et 641 sont très virulentes pour la plupart des animaux de laboratoire. Les souches 844, 1183, 1224, 1262, 967, 1208, 1220 sont de provenance humaine et furent prélevées chez des porteurs sains. La souche FDA 209 est une souche de laboratoire bien connue, peu virulente pour les animaux d'expérience et sensible à la pénicilline.

La substance antibactérienne à étudier est déposée, avant ou après l'injection des staphylocoques, sous forme de gouttes à la surface de la membrane coquillière qui tapisse la chambre à air. Si on veut étudier l'action d'une telle substance tout de suite après le moment de l'infection, on la dépose sur la membrane coquillière en évitant l'endroit où l'aiguille est passée pour l'injection de l'embryon. La substance diffuse assez rapidement dans tout le contenu de l'œuf pour y exercer ses effets. Si on veut étudier l'action d'une telle substance sur les phases plus avancées de l'infection, on peut sortir les

œufs de l'étuve de une à six heures après l'injection des staphylocoques. On entr'ouvre la fenêtre artificielle réalisée avec le cellophane collant (scotch tape) pour déposer les gouttes de substance antibactérienne sur le dédoublement intact de la membrane coquillière. On referme par la suite la fenêtre et on dépose une fois de plus les œufs à l'étuve. On pourrait aussi administrer cette substance par voie intraveineuse suivant une méthode déjà décrite pour la méthode d'inoculation du jaune d'œuf (4). D'autre part, on pourrait avoir intérêt à administrer la substance antibactérienne mélangée aux staphylocoques.

Résultats

(*1*) *Embryons Infectés et Non Traités Avec des Substances Antibactériennes*

Les embryons infectés et non traités avec des substances antibactériennes ont présenté comme prévu une infection grave, avec une mortalité voisine de 50% ou de 100% selon la dose utilisée (Tableaux I, II, III, et IV).

(*2*) *Action de la Pénicilline*

Dans une première série d'expériences, indiquées au Tableau I nous avons étudié l'action de la pénicilline sur des infections produites avec des souches différentes.

(*a*) *Souches Résistantes à la Pénicilline in vitro*

Les souches 1183, 1224, 844, et 1262 sont résistantes *in vitro* aux concentrations de 0.1, 1, et 10 unités par gramme. La mortalité des embryons inoculés avec les deux premières souches n'a pas été influencée par le traitement à la pénicilline. Ainsi, leur mortalité est pratiquement égale à celle des embryons infectés mais non traités (Tableau I).

Les souches 844 et 1262, malgré leur résistance *in vitro*, semblent sensibles à la pénicilline *in vivo* car la mortalité des embryons traités à la pénicilline est beaucoup plus faible que celle des non traités (Tableau I).

La souche 641 est résistante *in vitro* aux concentrations de 0.1 et de 1 unité mais elle est sensible à la concentration de 10 unités par gramme. *In vivo*, la pénicilline montre une action protectrice partielle. Tandis que les embryons non traités présentent une mortalité de 100%, les traités ont une survie de 50% (Tableau I).

(*b*) *Souches Sensibles in vitro à la Pénicilline*

Tous les embryons infectés avec des souches sensibles *in vitro* à la pénicilline (209 FDA, A8, 1208, et 1220) ont été entièrement protégées par cette dernière (Tableau I). La mortalité de 2 sur 20 rencontrée chez des embryons protégés est due à une mortalité résiduelle provoquée par le traumatisme dû à l'inoculation. Elle est rencontrée même chez des embryons témoins non infectés mais injectés avec de l'eau salée stérile.

(*c*) *Détermination de la Plus Faible Dose de Pénicilline à Utiliser par Œuf*

Dans une série d'expériences, nous avons voulu déterminer quelle était, par œuf, la plus faible dose de pénicilline capable de donner des résultats

TABLEAU I

SENSIBILITÉ À LA PÉNICILLINE DE QUELQUES SOUCHES DE STAPHYLOCOQUE *in vitro* ET *in vivo*

Souche	*In vitro*	Unités de pénicilline par œuf	Mortalité des embryons inoculés	
			Avec pénicilline*	Sans pénicilline
209 FDA	Sensible		2/20	16/20
A8	Sensible	500	2/20	20/20
641	Partiellement Résistante		10/20	20/20
1220	Sensible	50	2/20	20/20
1183	Résistante		20/20	20/20
1224	Résistante	50	19/20	20/20
844	Résistante	50	5/20	20/20
1262	Résistante	50	7/20	15/20
1208	Sensible		2/20	17/20

* *Administrée une heure après l'inoculation des staphylocoques.*

constants avec une souche sensible. Nous avons utilisé la souche A8 à des concentrations rapprochées de la dose minima mortelle. La pénicilline a été administrée à une ou à deux heures après l'inoculation des staphylocoques. En bas de 50 unités, nous avons obtenu des réponses irrégulières, mais avec 50 unités par œuf on obtient une protection constante (Tableau II). Avec la même souche, ces 50 unités de pénicilline par œuf ont protégé aussi des embryons injectés avec un inoculum équivalent à 100,000 LD_{50} ou à 100 fois la DMM.

TABLEAU II

EFFETS PRODUITS PAR DES DOSES DIFFÉRENTES DE PÉNICILLINE SUR L'INFECTION EXPÉRIMENTALE AVEC UNE SOUCHE SENSIBLE (A8) DE STAPHYLOCOQUES

Unités de pénicilline par œuf	Temps*	Mortalité des embryons inoculés	
		Avec pénicilline	Sans pénicilline
500	1 heure	1/20	20/20
50		2/20	
250	2 heures	5/20	15/20
500		3/20	
50		1/20	
250	2 heures	1/20	19/20
500		1/20	
5		7/20	
10	1 heure	15/20	20/20
250		0/20	
25		1/20	
10	2 heures	4/20	19/20
5		10/20	

* *Intervalle de temps écoulé entre l'injection des staphylocoques et l'administration de la pénicilline.*

(*d*) *Effet de l'Administration de la Pénicilline à des Intervalles Variables après l'Inoculation de Staphylocoques*

Nous avons voulu déterminer si notre méthode permettait l'étude de l'action d'une substance antibactérienne à des moments différents de l'infection. Nous avons utilisé la souche A8 à une concentration rapprochée de la dose L_{50} pour les deux premières expériences et à une concentration rapprochée de la dose minima mortelle dans les deux dernières.

La pénicilline s'est avérée efficace si on l'administre dans les cinq premières heures de l'infection expérimentale. Après six heures son action est encore appréciable (Tableau III).

TABLEAU III

EFFET PRODUIT PAR L'ADMINISTRATION DE LA PÉNICILLINE À DES INTERVALLES DE TEMPS VARIABLES APRÈS L'INOCULATION DES STAPHYLOCOQUES (SOUCHE A8)

Unités de pénicilline par œuf	Temps*	Mortalité des embryons inoculés	
		Avec pénicilline	Sans pénicilline
500	1 heure	2/20	13/17
	2 heures	2/20	
	4 heures	2/20	
	5 heures	3/30	
50	2 heures	0/20	11/20
	3 heures	1/20	
	4 heures	1/20	
	5 heures	1/20	
50	2 heures	1/20	19/20
	3 heures	4/20	
	4 heures	2/20	
	5 heures	2/20	
50	1 heure	3/20	20/20
	2 heures	1/20	
	3 heures	1/20	
	4 heures	2/20	
	5 heures	3/20	
	6 heures	7/20	

* *Intervalle de temps écoulé entre l'injection des staphylocoques et l'administration de la pénicilline. Pendant ce temps les œufs sont restés à 37° C.*

(3) *Action Antibactérienne de Deux Sulfamides Solubles*

Nous avons voulu déterminer si notre méthode se prêtait à l'étude d'autres substances que les antibiotiques. Nous avons choisi les substances sulfamidées, pour lesquelles les tests *in vitro* de la sensibilité des souches se font plus rarement en clinique, à cause des difficultés techniques.

Nos études ont été limitées à deux préparations solubles, de ce groupe, la sulfadiazine sodique, connue sous le nom commercial de Soludiazine*, et

le sulfathiazol, connu sous le nom commercial de Soluthiazol*. Une préparation de Soluseptazine*, substance réputée comme inactive contre les staphylocoques, a servi de témoin inactif.

Les quantités de ces substances administrées dans chaque œuf ont été calculées en poids pour représenter cinq fois la concentration utilisée en clinique.

Les résultats résumés dans le tableau IV montrent que cinq sur six des souches prises au hasard se sont montrées sensibles à une ou aux deux substances sulfamidées, ce qui est un résultat très encourageant en faveur de l'utisation de ces substances.

TABLEAU IV

DÉTERMINATION *in vivo* DE LA SENSIBILITÉ DE QUELQUES SOUCHES DE STAPHYLOCOQUE POUR DES SUBSTANCES SULFAMIDÉES

Souche	Mortalité des embryons inoculés			
	Sans traitement	S. diazine	S. thiazol	S. septazine
967	5/5	3/10	2/9	8/8
1220	4/4	1/10	2/8	9/9
A8	19/20	2/20	1/20	18/20
1220	20/20	2/20	13/20	20/20
1183	20/20	20/20	19/20	10/10
1224	20/20	13/20	4/20	10/10

Discussion et Conclusion

En modifiant notre méthode d'infection staphylococcique de l'embryon de poulet, nous avons mis au point une épreuve quantitative de l'action *in vivo* des substances antistaphylococciques. Pour la première fois, on a réussi à reproduire à volonté des mortalités voisines de 100% et de 50%. Contrairement aux méthodes décrites auparavant, on produit une infection mortelle en employant des nombres beaucoup moins considérables de bactéries.

Parmi les souches étudiées, celles qui se sont montrées sensibles à la pénicilline *in vitro* ont toujours été également sensibles *in vivo*. Les souches résistantes *in vitro* se sont partagées en deux catégories. Deux des souches étudiées ont été résistantes *in vivo* aussi, tandis que deux autres souches, malgré leur résistance *in vitro*, se sont comportées *in vivo* comme des souches sensibles à la pénicilline. Il y aurait intérêt à poursuivre des études cliniques sur ce sujet.

Un traitement de 50 unités de pénicilline par œuf semble suffisant. L'embryon est ainsi protégé même contre des doses infectantes cent mille

** Nous remercions la maison Poulenc pour avoir gracieusement mis ces substances à notre disposition.*

fois supérieures à la LD_{50}. L'efficacité de la pénicilline reste la même au cours des six premières heures de l'infection expérimentale, quel que soit le moment où on l'administre.

Deux sulfamidés solubles ont protégé les embryons contre l'infection avec la majorité des souches essayées. Il serait intéressant de rechercher la portée clinique de tels essais.

Le fait que notre méthode peut utiliser des doses infectantes réduites qui donnent naissance à un envahissement progressif de l'embryon, sans toxémie initiale, nous permet d'intervenir avec les substances antibactériennes sur une infection expérimentale qui rappelle dans ses débuts l'évolution de la plupart des staphylococcies humaines. Elle s'en rapproche aussi par le fait que les globules rouges des embryons de poulet présentent une résistance aux toxines staphylococciques égale à celle des globules rouges humains.

Nous sommes en train d'étudier l'action des anticorps sur cette infection expérimentale en mettant à profit les avantages qu'elle offre.

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THE CHEMICAL COMPOSITION OF THE CELL AND CELL WALL OF SOME HALOPHILIC BACTERIA[1]

By W. R. Smithies[2], N. E. Gibbons[3], and S. T. Bayley

Abstract

Cells of the halophilic bacteria *Micrococcus halodenitrificans*, *Vibrio costicolus*, and *Pseudomonas salinaria* have a relatively high nitrogen content, indicating that the cell material is predominantly protein. They contain only small amounts of fat and no detectable amount of carbohydrate other than nucleic acid pentose. Cell walls are lipoprotein. Cells disrupted by shaking with water alone contained electron-dense material similar in composition to the cell walls. In a few preparations of the *Micrococcus* a polymer of β-hydroxybutyric acid was found.

Introduction

Early work on the chemical composition of bacteria and the nature of their cell walls, as reviewed by Knaysi (5), and extensive studies by Salton (15) have demonstrated the diversity in the composition of the cell walls of different bacterial species. In general they contain protein, carbohydrate, and variable amounts of lipid. Occasionally glycerophosphates and mucoid substances are present.

Recent work on halophiles (1, 13, 14) has indicated that an energy mechanism is most probably responsible for the selective permeability of the outer membrane. However, little is known of the chemical composition of the cells and cell walls of halophiles and there was a possibility that the ability of these organisms to grow in high salt concentration may be related to the chemical composition of the cells. Studies were therefore undertaken on the cellular composition of two moderate halophiles, *Vibrio costicolus* and *Micrococcus halodenitrificans*, and the extreme halophile *Pseudomonas salinaria*.

Materials and Methods

Organisms

The strains of *Pseudomonas salinaria*, *Vibrio costicolus*, and *Escherichia coli* were those used by Baxter and Gibbons (1) and, unless otherwise stated, the media and methods of cultivation used were the same. *Micrococcus halodenitrificans* was grown as described by Robinson and Gibbons (13). Cultures of *P. salinaria* and *M. halodenitrificans* in volumes greater than 200 ml. were aerated or shaken, the latter method usually producing a greater yield of cells. Cells were harvested towards the end of the logarithmic phase of growth by centrifugation and washed twice with aqueous sodium chloride of the same concentration as in the medium.

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 3705.
[2] *National Research Council of Canada Postdoctorate Fellow 1953–1954, 1954–1955.*
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Preparation of Cell-wall Material

Shaking cell suspensions with fine glass beads (ballotini No. 12)* was of value in the preparation of cell-wall material from *M. halodenitrificans* and *P. salinaria*, but completely fragmented the cell walls of *V. costicolus*. The *Micrococcus* cells, after having been washed once with 4% sodium chloride and once with water, were suspended in water (*ca.* 30 mgm. dry weight/ml.) with 80 gm. beads in a total volume of 100 ml. in a 150-ml. bottle. A trace of deoxyribonuclease was added to each bottle to reduce the viscosity resulting from the liberation of cell contents. The bottle was then shaken for four to five hours at approximately 250 cycles per minute at a temperature of 1–4° C. *P. salinaria* cells were washed with and shaken in 20% sodium chloride.

Shaking with water alone should suffice to disrupt the cells effectively, as the cells of halophiles plasmoptize in water. However, in heavy concentrations (10–20 mgm./ml. dry weight) of cells, a viscous suspension was formed and only a portion was broken up unless the viscosity was reduced by the addition of deoxyribonuclease. When the enzyme was added, shaking with water produced relatively undamaged cell walls of *V. costicolus* and of *M. halodenitrificans*. With *P. salinaria* however the cell substance was completely dispersed and no particulate material could be recovered.

Treatment of suspensions of halophilic cells in water with a 10 kc. "Raytheon" sonic vibrator was not generally satisfactory. Exposures of 5 to 20 min. at 1.0 to 1.1 amp. broke up a high percentage of cells but, on examination with the phase contrast and the electron microscope, the cell walls were found to be completely fragmented and could not be distinguished from cytoplasmic debris.

Cell walls were isolated from disrupted cells by the methods described by Salton (15). Ballotini, when used, were removed by filtration through coarse sintered glass, and the unbroken cells by centrifugation at 1500 × *g*. The particulate cell-wall material was separated from the supernatant by centrifuging at 12,000 × *g* for 10–20 min. For the moderate halophiles, the sediment was washed once with 1.0 *M* sodium chloride solution, then three times with water and suspended in water to give a stable dispersion. Further washings may well have removed contaminating cytoplasmic material, but invariably, after four or five washings the product aggregated and could no longer be dispersed. The viscous red product of disruption of *P. salinaria* cells gave a pale-pink sediment on centrifugation at 1500 × *g*, and a bright-red sediment of cell walls at 12,000 × *g* for 20–30 min. The cell walls were washed four times with, and then resuspended in, 20% sodium chloride to form a stable dispersion.

Chemical Methods of Analysis

Dry weight was determined after heating at 100°–110° C. overnight, and ash content after heating at a dull red heat to constant weight. Care was

* *Chance Bros., Smethwick, England.*

taken to avoid fusing the salt residues. Chloride was estimated (as sodium chloride) by the method of Schales and Schales (17) and all results are expressed on a salt-free basis.

Nitrogen was determined by the micro-Kjeldahl method using red mercuric oxide – potassium sulphate mixture as a catalyst; phosphorus, by a modification of the method of Kuttner and Lichtenstein (6).

Carbohydrate was estimated by hydrolyzing with 2 *N* hydrochloric acid for two hours at 100° C., as suggested by Salton (15), and measuring the reducing substances by the method of Nelson (11) as modified by Somogyi (19). Pentose estimations on the hydrolyzates were made by Brown's modification of the method of Mejbaum (2). The total carbohydrate content of cell material was also determined by measuring the intensity of the color given when the material was heated with orcinol (12).

The total nucleic acid content of cell suspensions was estimated by the measurement of optical density at 260 mμ and 350 mμ as described by Mitchell (8) using a Beckman model DU spectrophotometer, or a Cary Recording Spectrophotometer, model 11M. Deoxyribonucleic acid was extracted from cell suspensions with 5% trichloracetic acid (18) and determined with the diphenylamine reagent (3).

Total fatty material was estimated gravimetrically as ether-soluble material after hydrolysis with 6 *N* hydrochloric acid for two hours at 100° C. (15).

Results

Composition of Cells of Halophiles

Compared with non-halophiles, the cells of halophilic bacteria have a relatively high nitrogen and low carbohydrate content (Table I). The orcinol method confirmed the low carbohydrate values. It was found that 75–90% of the total reducing substance was pentose in nature and only ribose could be detected by paper chromatography. It seems very likely, therefore, that the halophile cells examined contain little carbohydrate other than nucleic acid pentose. If it is assumed that the bacterial nucleic acid contains 45% pentose, and that only 45% of this is estimated by the procedures used (4), a pentose nucleic acid content of 15% would give rise to 3.0 gm. pentose per 100 gm. dry weight of bacterial cells, which is in agreement with the figures obtained.

No increase in the carbohydrate content of the cells could be detected when the *Vibrio* and *Micrococcus* were grown in media to which 0.5% glucose had been added.

The high nitrogen content and strongly positive Biuret reaction of the halophile cells indicate that protein is a major constituent. The fat content is below 10% which, according to Knaysi (5), is normal for bacteria that do not deposit ether-soluble lipid inclusions. The relative amounts of nucleic acid and total phosphorus show that other phosphorus-containing compounds cannot be present in large amount.

TABLE I

COMPOSITION OF CELLS OF HALOPHILIC BACTERIA

(Expressed as gm./100 gm. dry weight (NaCl free))

Organism	Total nitrogen	Total phosphorus	Carbohydrate*	Total nucleic acid	D.N.A.	Total fat
V. costicolus	13.5	2.5	3.0	25.0	3.0	9.0
M. halodenitrificans	13.0	2.2	3.0	18.0	2.5	5.9
M. halodenitrificans (0.15% $CaCl_2$ in medium)	11.5		3.5	16.0	3.4	6.0
P. salinaria	12.7	2.1	3.0	15.0	4.3	4.0
Average values for non-halophiles (Knaysi)	6.3–11.4		10.0–30.0	10.0–30.0		

NOTE: *Cells harvested towards end of logarithmic phase of growth.*
** Determined as reducing substance after hydrolysis.*

Nature of the Cell Walls

Electron Microscopic Examination

Electron micrographs of various cell-wall preparations of halophiles are shown in Figs. 1 to 6. Practically all are circular or elliptical in shape, whether prepared from cocci (Figs. 3 to 6), rods (Fig. 1), or vibrios (Fig. 2).

The *P. salinaria* micrograph (Fig. 1) prepared from suspensions in 20% sodium chloride shows many electron-dense salt crystals. The electron-dense cytoplasmic material in the vibrio preparation (Fig. 2) could not be removed by washing, and one cell wall is shown which still contains electron-dense material.

Coccus cell walls prepared by shaking with ballotini beads (Figs. 3*A* and *B*) were relatively whole, thin, and collapsed. Some cytoplasmic debris was present outside the cell. Cell walls prepared by shaking with water alone, a relatively gentle procedure, contained electron-dense cytoplasmic material within many of the cell envelopes (Fig. 4).

Cell walls of cocci grown in media to which 0.15% calcium chloride was added, and disrupted by shaking with ballotini, were thicker and had a rougher surface structure (Fig. 5) than those grown without calcium (Figs. 3*A* and *B*). When disrupted by shaking with water alone, practically all the cell walls contained electron-dense material (Fig. 6), indicating that calcium increased the strength of the cell wall and/or cytoplasmic membrane. Cell walls in this

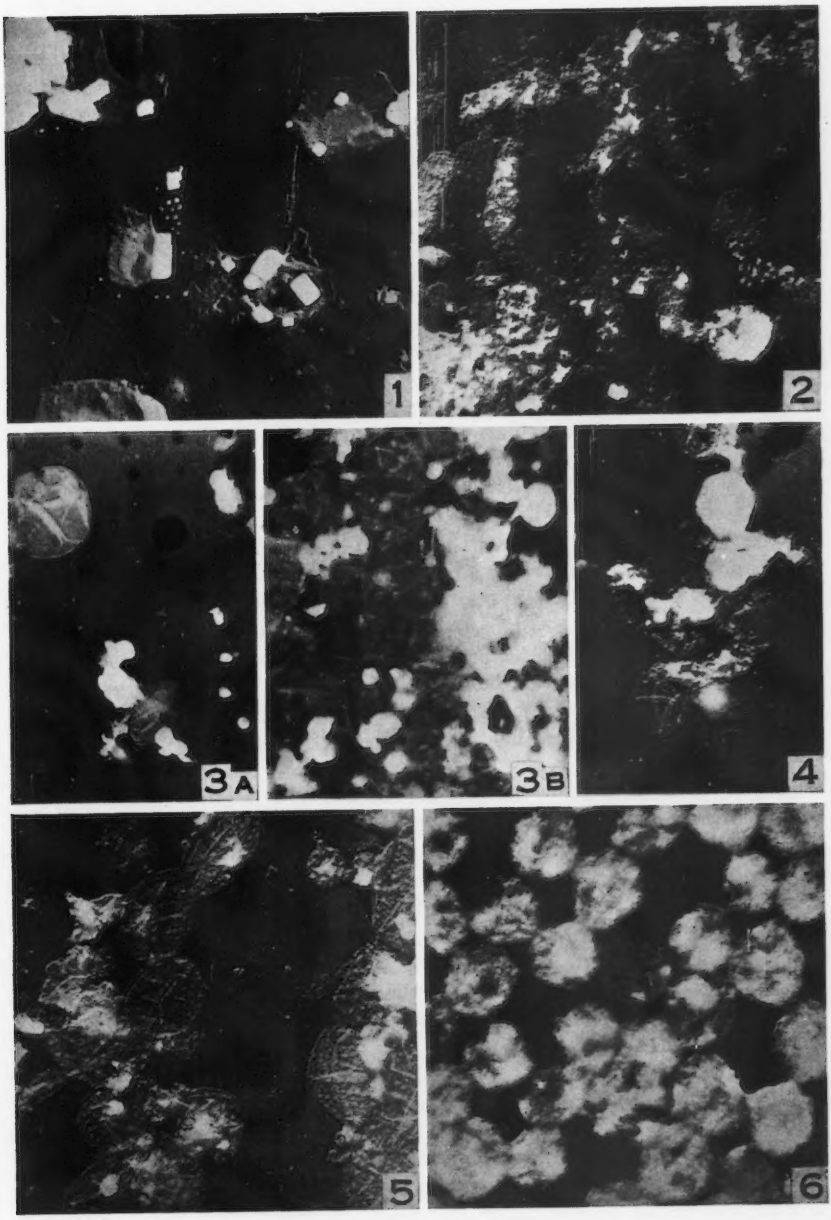
FIGS. 1–6. Electron photomicrographs of cell walls. Chromium shadowed. Magnification *ca.* × 7000.

FIG. 1. *P. salinaria*, shaken in 20% sodium chloride.

FIG. 2. *V. costicolus*, shaken in water.

FIGS. 3–6. *M. halodenitrificans.* FIGS. 3*A* and 3*B*. Shaken in water with ballotini. FIG. 4. Shaken in water alone. FIG. 5. Cells grown in presence of 0.15% calcium chloride, shaken in water with ballotini. FIG. 6. Cells grown in presence of 0.15% calcium chloride, shaken in water.

PLATE I



preparation were relatively heavy and spun down completely at 6000 × *g*, instead of the usual 12,000 × *g*. The number of unbroken and viable cells was therefore greater in such preparations but in the heavy suspensions used constituted only a trace contamination.

Composition of Cell Walls

Cell-wall preparations of both moderate and extreme halophiles contained relatively large amounts of nitrogen and of fat (Table II). In this respect they resemble most closely the lipoprotein cell walls of *E. coli* (15) and may be assumed to be of a similar nature. Only traces of carbohydrate could be detected.

Cell walls of *M. halodenitrificans* prepared by sonic vibration contained a much lower percentage of nitrogen than other preparations of this organism. If the nitrogen is assumed to be present as protein, as indicated by a positive Biuret reaction, the analysis accounts for less than 40% of the dry matter. After the cell-wall preparation had been boiled with 6 *N* hydrochloric acid, a yellow fatty material was extracted from the residue with chloroform. When the residue was washed with ether, a white powder was obtained which was found to contain 55.5% C and 7.3% H. This was tentatively identified as the polyester of β-hydroxybutyric acid, reported in other bacteria (7, 20), which has the empirical formula C_2H_3O and requires 55.8% C and 7.0% H. A study of the degradation product of the polymer from the *Micrococcus* confirmed its identity. After hydrolysis of the polymer with 2 *N* sodium hydroxide, ether extraction of the acidified solution yielded a product with the characteristic odor of crotonic acid, which is formed under the conditions of the hydrolysis from β-hydroxybutyric acid through loss of water. The ester formed with *p*-bromphenacyl bromide had a melting point of 95° C.; when mixed with the ester prepared from authentic crotonic acid the melting point was unchanged.

The polymer could also be isolated from intact cells by treating them with 0.2 *N* sodium hydroxide overnight at room temperature and washing the white residue with 0.2 *N* NaOH, dilute acetic acid, and finally ethanol. The polymer was separated from the residue by extraction with chloroform. The yield was about 3% of the dry weight of the cells, whereas in *B. megaterium* it may constitute over 26% of the dry mass (7). With the *Micrococcus*, sonic vibration apparently separates the polymer from the cytoplasm and the polymer fragments sediment with the cell-wall material.

The absence of an absorption peak or pronounced inflection at about 260 mμ indicated the lack of all but traces of nucleic acids in the cell-wall preparations of *M. halodenitrificans* (Fig. 7). The curves obtained with cell walls of *V. costicolus* were nearly identical. With *P. salinaria* cell walls there was an absorption peak around 270 mμ but this is the result, probably, of pigment associated with the cell wall. When the pigment together with the low molecular weight substances was extracted from the cells with ethanol, the red solution had a similar absorption peak. In *P. salinaria* cells the absorption peak at 260 mμ, due to nucleic acid, predominates (Fig. 7).

TABLE II

COMPARISON OF COMPOSITION OF CELL WALLS OF HALOPHILIC AND NON-HALOPHILIC BACTERIA

(Expressed as gm./100 gm. dry weight)

Organisms	Method of cell disruption	Total nitrogen	Total fat	Total phosphorus	Carbohydrate*	Ash
V. costicolus	Sonic vibration	14.2	10.1	0.53	1.5	—
	Shaken with beads	13.8	11.8	—	1.6	—
	Shaken with water	12.8	19.9	1.3	1.5	3.6
M. halodenitrificans	Sonic vibration	4.4	6.0	0.4	0.5	2.0
	Shaken with beads	12.3	12.5	—	—	—
	Shaken with water	13.5	18.0	1.1	—	—
M. halodenitrificans	Shaken with beads	12.2	18.0	0.8	—	—
(0.15% $CaCl_2$ in medium)	Shaken with water	14.6	10.0	—	—	4.7
P. salinaria†	Shaken with beads	11.0	11.0	1.0	—	—
E. coli	Sonic vibration	10.0	21.0	1.2	15.0	
E. coli‡		10.0	22.6	1.52	16.0	
S. pullorum‡		6.4	19.0	0.88	46.0	
S. lutea‡		7.6	1.1	0.22	46.5	
S. pyogenes‡		10.6	—	0.62	33.1	
B. subtilis‡		5.1	2.6	5.35	34.0	

* *Determined as reducing substance after hydrolysis.*
† *Results given on salt-free basis.*
‡ *As determined by Salton (15).*

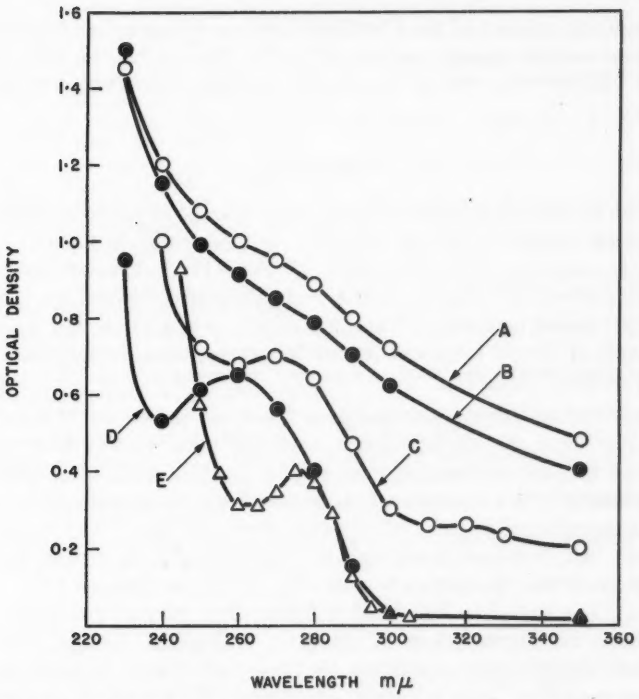


FIG. 7. Absorption spectra of halophilic cell components. Cell walls of *M. halodenitrificans*—(A) grown with 0.15% added $CaCl_2$, shaken with water alone, and contained considerable electron-dense material; (B) grown without added $CaCl_2$, shaken with ballotini, and contained little electron-dense material: *P. salinaria* cell walls (C), disrupted cells (D), and pigment (E).

The electron-dense material found in cell-wall preparations probably includes the cytoplasmic membrane and adhering precipitated cytoplasmic protein. Preparations containing both large and small amounts of electron-dense material were composed of protein and fat (Table II), free from nucleic acid (Fig. 7).

With cells grown in the presence of calcium the yield of more or less empty cell walls of the *Micrococcus*, prepared by shaking with ballotini (Fig. 5), was about 5% of the dry cell weight, whereas the yield of cell walls plus cytoplasmic material, prepared by shaking with water alone (Fig. 6), was nearly 30%. This would indicate that in the later case, considerable cytoplasmic material was involved which contained more nitrogen and less fat than the relatively empty cell walls (Table II). With cocci grown in the absence of added calcium, the yield of cell-wall material prepared by shaking with water alone was about twice that obtained with ballotini. This agrees with electron microscope observations which showed that one-third to one-half of the envelopes prepared by the former method (Fig. 4) contained electron-dense material.

The high salt content of the *P. salinaria* cell-wall dispersions made accurate estimations on the organic matter difficult. Except for the slightly lower nitrogen content the results are similar to those obtained for the other halophiles.

Discussion

The lack of carbohydrate in the cell and cell walls of the halophiles studied is an unusual feature. The cell wall of *E. coli*, examined by Salton (15), most closely resembles that of the halophiles (*cf.* Table II), but it contains a definite carbohydrate moiety, yielding glucose and galactose on hydrolysis. Although Weidel (21) found no carbohydrate in a similar preparation, the composition of cell walls of *E. coli* prepared in our laboratory was very similar to that given by Salton (Table II).

The cell wall of *Micrococcus pyogenes* has been shown by Mitchell (9) to contain less than 2% carbohydrate, together with 10.6% total nitrogen, 4.4% total fat, and (calculated from results given) 2.52% total phosphorus. Predominantly, it is a complex of protein with glycerophosphate from which the total phosphorus content is almost entirely derived. The halophile cell walls have not, however, been examined specifically for glycerophosphates since the analytical figures show that they could be present only in trace amounts. The halophile cells had a lower total phosphorus and a higher ether-soluble fat content than *M. pyogenes* or *Bacillus subtilis* (these latter two being similar in composition (9, 15)). In these characteristics the halophiles resemble Gram-negative organisms (15, 10) which, according to Mitchell (10), are more or less free from glycerophosphates but rich in phospholipids, from which the phosphorus content of the halophile cell walls may be derived.

The lipoprotein cell walls of *V. costicolus* and *P. salinaria* are very fragile, probably more so than the cell walls of most non-halophiles, but the ease with which good preparations are obtained from the *Micrococcus* indicates, however, that there is nothing inherently weak in lipoprotein cell walls. In contrast with the cell walls of *Bacillus megaterium* and *Rhodospirillum rubrum* (16), the halophilic cell walls are fragmented by sonic vibration.

All the cell-wall preparations were contaminated with electron-dense material, and the micrographs (Figs. 2, 4, 6) show that under suitable conditions of cell disruption this 'debris' may remain inside the cell envelope forming a thick, rugose layer, the appearance of which agrees well with Knaysi's description of the stained cytoplasmic membranes of bacilli (5). However, Knaysi's interpretations have been questioned and it may be that the electron-dense material includes a preponderance of precipitated cytoplasmic protein.

Although halophilic cells and cell walls do differ in composition from those of non-halophiles, particularly with respect to their nitrogen and carbohydrate contents, no relation can be seen as yet between this and the phenomenon of halophilism.

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THE DEOXYRIBOSE NUCLEIC ACID SLIME LAYER OF SOME HALOPHILIC BACTERIA[1]

By W. R. Smithies[2] and N. E. Gibbons[3]

Abstract

Cells of *Micrococcus halodenitrificans* and *Vibrio costicolus* when grown under certain conditions possess a sticky extracellular deoxypentose nucleic acid (DNA) slime. Treatment of these cells with deoxyribonuclease (DNase) eliminates the stickiness and removes up to 50% of the DNA of the cells, without affecting their viability. A similar DNA slime is probably responsible for the stickiness of *Pseudomonas salinaria* and other halophiles, and the presence of such a slime seems peculiar to halophiles. The stickiness of *M. halodenitrificans* and the amount of DNA extractable from it with DNase decrease with increasing salt content of the growth medium, or if calcium is added to the medium. The results suggest that a deficiency of essential nutrients affects the permeability of the membrane allowing leakage of DNA or its precursors from the cell.

Introduction

Many halophilic bacteria produce a characteristic viscous* growth in liquid and on solid media. The stickiness of the cells makes many quantitative aspects of their study difficult, and centrifuged cells are practically impossible to disperse by ordinary methods. In non-halophilic bacteria, slimy extracellular layers, when present, are usually polysaccharide, but peptide and lipid slimes are also known (12, 15). In an earlier paper (21) it was pointed out that halophilic cells contain little carbohydrate other than nucleic acid pentose. It was conjectured therefore that the slime was not polysaccharide and might well contain a high percentage of nitrogen. However, it was with some surprise that it was discovered that deoxyribonuclease not only reduced the viscosity of liberated cytoplasm, but also completely removed the stickiness and slime associated with viable cells. This paper deals with some aspects of this phenomenon.

Materials and Methods

Micrococcus halodenitrificans, *Vibrio costicolus*, *Pseudomonas salinaria*, and a very sticky, colorless, unidentified, extreme halophile (strain A2c), found associated with a red halophile obtained from Norway, were used as representative halophilic bacteria. Cultural methods for these organisms have been described (3, 17). The non-halophiles *Micrococcus denitrificans* (22), *Micrococcus freudenreichii* No. 325 (1), and *Aerobacter aerogenes* isolated from ropy milk (11) were used for comparison. *M. denitrificans* was grown in

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* *Viscous and sticky are used synonymously in this paper. Not only do the cells stick together but the mass may be drawn out in fine threads when touched with a needle. However, only the cells are sticky; the broth itself is not viscous as long as the cells remain whole.*

PPT broth (0.5%* proteose peptone, 0.5% tryptone) containing 0.085% sodium nitrate and 2% sodium chloride (this amount of salt approaches the top limit for growth of this organism); *M. freudenreichii* in PPT plus 1.0% glucose and 0.9% salt; *A. aerogenes* on PPT agar plus 1% milk.

Viable cell counts of the halophiles were determined by the "drop-plate" procedure on agar medium containing 4% sodium chloride (4). Occasionally growth was estimated by turbidity measurements at 660 mμ.

Chemical methods, unless otherwise mentioned, have been described in a previous paper (21). Deoxyribonuclease, crystallized once; ribonuclease, crystalline (salt-free); and trypsin, crystallized twice (salt-free), were obtained from General Biochemicals, Inc.; purified lysozyme from Armour and Co.

Estimation of Nucleic Acids

Total nucleic acids (NA)† in cell suspensions were estimated by measuring the optical density at 260 mμ and correcting as described by Mitchell (14) but taking $E^{1\%}_{1cm.}$ as 250 (8). The optical density at 260 mμ, without correction, was used to estimate NA in cell-free extracts. These methods were used rather than the phosphorus precipitation methods since the samples frequently contained partially degraded nucleic acids.

Cell suspensions were extracted with 5% trichloroacetic acid (19) and DNA was estimated on the extracts with the diphenylamine reagent (6). A sample of DNA prepared from calf thymus by the method of Mirsky and Pollister (13) and deproteinized by the method of Sevag *et al.* (20) was used as standard. Since the low sensitivity of the diphenylamine reagent is accentuated in the presence of sodium chloride, it was essential that standards and unknowns contained identical final salt concentrations.

Removal and Isolation of Extracellular Nucleic Acids

To isolate undegraded DNA from the slime layer, cells were shaken with 0.5% sodium deoxycholate in 4% sodium chloride at 1–4° C. for 60 min., and then centrifuged at 2000 × *g* (2). The addition of two volumes of 95% ethanol to the viscous supernatant gave a fibrous precipitate, which was collected on a glass rod by stirring the mixture. The precipitate was dissolved in 5.85% sodium chloride, freed of the small amount of insoluble material by centrifugation, and reprecipitated with ethanol. Purine and pyrimidine components of the DNA were detected by paper chromatographic analysis, using the methods of Wyatt (23).

The extracellular nucleic acids could be removed more or less quantitatively by enzymic treatment. About two grams of wet cells were washed and resuspended in 10 ml. of 4% sodium chloride. Extracellular DNA was degraded by adding a large excess of DNase (about 0.1 mgm.) and 0.4 ml.

* *Per cent expressed on W/V basis unless otherwise noted.*

† *The following abbreviations are used in this paper: NA—total nucleic acids; DNA—deoxypentose nucleic acids; RNA—ribose (more strictly pentose) nucleic acids; DNase—deoxyribonuclease: RNase—ribonuclease.*

0.075 *M* magnesium sulphate, and allowing the suspension to stand at room temperature 1.5 to 2 hr. with occasional stirring. To remove RNA as well, 0.1 mgm. of RNase was added after 30 min. In earlier experiments the sample was centrifuged at 2000 × *g* for 15 min. and the amount of DNA and RNA extracted estimated on the supernatant. In later experiments the amount removed was estimated by difference from determinations on the suspension after enzymic treatment and on the cells after centrifuging, washing with 4% sodium chloride, and resuspending in their original volume. Whenever estimations on cells or extracts were compared directly, care was taken that the samples contained equivalent concentrations of nucleases (where appropriate) and salt; and such estimations were made, as far as possible, at the same time.

Experimental and Results

The sticky cells of halophiles are difficult to disperse, particularly after centrifugation. In a veronal or phosphate buffer of pH 7.5, containing 0.003 *M* magnesium sulphate and 4% sodium chloride, the addition of as little as 4 μgm. of DNase per milliliter allowed sticky washed cells of *M. halodenitrificans* and *V. costicolus* to be dispersed readily. RNase (1 mgm./ml.), lysozyme (0.5 mgm./ml.), or trypsin (0.5 mgm./ml.) had no such effect and did not remove the stickiness. The DNase effect was inhibited by fluoride (0.1 *M*), citrate (0.01 *M*), or by heating to 60° C., while the addition of manganous ion to the citrate solution reactivated the enzyme. Dispersal of the cells is therefore a manifestation of DNase activity and the stickiness must be due to extracellular DNA.

While the presence of 4% sodium chloride does not affect DNase appreciably, the enzyme is inactivated by 20% salt and it is not possible, therefore, to show that the slime layers of the extreme halophiles can be removed by DNase. However, the enzyme completely removed the viscosity of the solutions obtained when the cells of *P. salinaria* and an unidentified species (A2c) were suspended in water, thus rupturing the cells. It is concluded that their extracellular stickiness, which must contribute to the total viscosity, is also due to the presence of DNA.

Part of the extracellular DNA could be recovered from the slime layer of *V. costicolus* by shaking with sodium deoxycholate but no such sticky material could be collected from cells treated beforehand with DNase. When the air-dried product was hydrolyzed, paper chromatographic analysis showed four spots, visible in ultraviolet light, corresponding to the four bases, guanine, adenine, cytosine, and thymine, obtained by hydrolysis of an authentic sample of calf thymus DNA.

An analysis of the material removed from cells of *M. halodenitrificans* by 3.5% sodium chloride alone, or salt solution containing DNase, or DNase plus RNase, is shown in Table I. These cells were grown with shaking in media containing 3.5% salt, harvested, and stored three days at 1–4° C. before treatment. The preparation was very sticky and the proportion of

TABLE I

ANALYSIS OF MATERIAL REMOVED FROM *M. halodenitrificans* BY DNASE AND RNASE

Extracted by	Grams of each component removed per 100 gm. of component present in cells					
	Dry weight	Total N	Total P	Pentose sugar	Total N.A.*	D.N.A.*
3.5% NaCl	11.0	12.0	15.0	16.6	11.5	8.0
+ DNase	24.0	23.5	32.0	34.6	25.0	51.0
+ DNase and RNase	30.0	29.7	42.0	50.0	40.0	46.0

* *Estimated from measurements on the extract (see text).*

TABLE II

THE EFFECT OF DNASE PLUS RNASE ON THE AMOUNT OF NUCLEIC ACID EXTRACTED FROM CELLS OF HALOPHILES AND NON-HALOPHILES BY SALT SOLUTIONS

Organism	Appearance	Grams of DNA and NA removed per 100 gm. of DNA and NA present in cells			
		Without nucleases		With nucleases	
		DNA	NA	DNA	NA
M. halodenitrificans	Sticky	< 3.0	9.2	27.0	16.6
M. halodenitrificans (0.15% $CaCl_2$ in medium)	Non-sticky	< 3.0	< 1.0	< 4.0	< 3.0
V. costicolus	Sticky	< 3.0	6.7	14.0	9.9
M. denitrificans	Non-sticky	< 3.0	< 1.0	< 3.0	< 2.0
E. coli	Non-sticky	< 2.0	< 2.0	< 3.0	< 3.0
M. freudenreichii	Non-sticky	< 1.0	5.5	< 3.0	36.2

Halophiles were extracted in 4%, and non-halophiles in 0.9% sodium chloride solution. All measurements were made on the extracts.

DNA removed relatively high; significant amounts of DNA usually could not be detected in non-enzymic extracts* by the diphenylamine reagent (Table II). DNase caused a preferential removal of DNA from the cells, and the amount was not increased by the addition of RNase. Some RNA was extracted when DNase was used and the amount was increased by the addition of RNase. The ultraviolet absorption spectra of the extracts were typical of nucleic acids. The relative amounts of phosphorus and pentose sugars removed agreed well with those for the nucleic acid content of the extract. Although these enzyme extracts gave a positive biuret test for

**Most of the nucleic acid is removed from the exterior of the cell and probably very little is actually extracted from the interior of the cell. Extract (extracted, etc.) is used in this sense throughout the paper.*

protein and also contain RNA, only DNase was capable of reducing the viscosity, and the material may therefore be presumed to be a DNA slime.

The amount of DNA which may be removed from cells of *M. halodenitrificans* by treatment with DNase decreases as the salt concentration of the medium in which the cells were grown increases. Cells grown in 2.5% salt were very sticky and 50–70% of the total DNA in the cells could be removed; cells grown in 4% salt were moderately sticky and only 10–15% of the total DNA could be removed; cells grown in 8% salt were not sticky and 5% or less of the total DNA could be removed. Because of the stickiness of cells grown in 2.5% salt, growth curves at this salt concentration were rather irregular. Growth curves of cultures in 4 and 8% salt, as determined by viable counts and turbidity measurements, were practically indistinguishable over periods up to six weeks, and during this time there was no indication of any decrease in cell numbers. Furthermore the amount of DNA which could be removed did not increase with time, at least for the three weeks over which tests were made with 4 and 8% cultures and for six weeks with 2.5% cultures. Although these results were based on only two experiments and are therefore not reported in further detail, they indicate that there is little autolysis and that the viscosity does not seem to be the result of cell breakdown. There does seem to be an association between the viscosity of the cells and the salt content of the environment.

When 0.15% calcium chloride was added to the medium, young cells of *M. halodenitrificans* grown in 3 and 4% salt were non-sticky and readily dispersed. Treatment with DNase did not remove a significant amount of DNA from such cells or from the cells of several non-halophiles (Table II). RNase extracted little RNA from the cells of *M. halodenitrificans* grown in the presence of calcium, or from the Gram-negative *E. coli* and *M. denitrificans* (22). Considerable quantities of RNA were extracted from the Gram-positive *M. freudenreichii*, as might be expected from the work of Henry and Stacey (9).

The results of the foregoing experiments indicated that the action of DNase was confined to the extracellular layers of the cell. This conclusion was supported by the fact that the viable count of cell suspensions was not reduced by treatment with DNase but sometimes even increased because the reduced viscosity allowed better dispersion. For example, the logarithm of the number of organisms present in a suspension of *M. halodenitrificans* in 4% sodium chloride incubated overnight with and without DNase was 9.04 and 7.83, and of *V. costicolus*, 9.50 and 9.31, respectively. Furthermore, it was shown, using the Warburg apparatus, that the rate of oxygen uptake by an actively growing culture of the coccus was unaffected by the addition of DNase or RNase.

One property of the slime layer is to protect halophilic cells from dehydration. Suspensions of *M. halodenitrificans*, containing 50 million cells per milliliter, were allowed to dry at room temperature. At the end of seven days the untreated cultures were reduced to a small amount of very viscous slime,

whereas the cells treated with DNase, as well as non-viscous cells grown in the presence of 0.15% calcium chloride, appeared dry. When diluted to the original volume with 4% sodium chloride, the viscous slime still contained 100,000 viable cells per milliter, while the dry residue contained less than 10 cells per milliliter.

Discussion

It is generally accepted that DNA is confined to the nucleus of the cell (5). However, Overend *et al.* (16) found that a saline extract from a virulent strain of *Hemophilus pertussis* contained DNA although autolysis did not occur under the conditions employed. Recently, a Feulgen staining layer on the outside of cells of *Azotobacter agile* has been reported (7), but in neither of these papers is definite evidence provided for the presence of an extracellular layer of DNA. It has been reported that the slime of *Aerobacter aerogenes*, associated with ropiness in milk, contained pentose, phosphorus, and purine (11), but since the viscosity of the cell mass is not reduced by treatment with DNase, the material is not comparable with the extracellular material of the halophiles.

Definite DNA slime layers have not been reported for non-halophiles and it is probable that they occur only in halophilic bacteria. DNase activity is low or negligible in halophilic cultures, but this is true apparently of many non-halophilic bacteria as well (18), and although the absence of DNase activity may be necessary for the formation of such slime, the absence of DNase cannot be used to explain the difference between halophiles and non-halophiles.

The slime layer of halophiles is apparently relatively thin. Centrifugation studies indicated that treatment with DNase decreases the cell volume of the sticky cells about 50%. The diameter of sticky cells, as seen in the phase microscope, is about 1μ, so that, if the intercellular space is neglected and the same type of packing assumed, the theoretical diameter of the slime-free cell would be about 0.8μ. Although this value is only an approximation, it indicates that the slime layer may be about 0.1μ thick. Attempts to stain the extracellular slime by the Feulgen method were not successful. The preliminary acid treatment may therefore have removed all, or most, of the DNA, although various concentrations of acid and times of hydrolysis were used. It is also possible that the concentration of DNA in this layer was not sufficient to be detected with this reagent, since only a fraction of the total cellular DNA is distributed over the whole cell, whereas in nuclear material most of the DNA is concentrated in a small volume.

With the *Micrococcus*, the amount of slime and of extracellular DNA decreases as the salt concentration of the medium increases. This decrease cannot be explained on the basis of the solubility of DNA, since, although the DNA isolated is soluble in 1 and 2 *M* salt solutions, neither the cell mass nor supernatant of cultures grown in 8 and 16% salt is viscous and no DNA-like material can be precipitated from the supernatant by ethanol. The increase

in viscosity in low salt concentration does not seem to be related to differences in the state of hydration of the DNA in the various salt solutions, since sticky cells from 2.5% salt remain sticky when transferred to 8% salt, and cells from 8% remain non-sticky for some time when transferred to 2.5% salt. With the coccus, therefore, the amount of slime seems to depend on the amount of DNA actually formed extracellularly. Although detailed examinations of the *Vibrio* have not been made, the relative effect of salt concentration and slime production is certainly not as clear cut, since cells of *V. costicolus* are sticky in salt concentrations ranging from 8 to 20%.

The presence of extracellular DNA cannot be explained by supposing it to be derived entirely from autolyzed cells. The viscosity of the medium is increased when cells are disrupted mechanically (21), and a similar effect would be expected when cells autolyzed. However, since the viscosity is confined to the cell mass, since young cultures are sticky, and since there is little evidence of a decrease in viable cells even in cultures several weeks old, autolysis does not offer a satisfactory explanation.

Although DNA slime has been found only in halophiles, the functional relation (if any) of these slimes to halophilism is not known. Since non-sticky cells can be grown, and DNase treatment does not affect viability, the slime cannot be involved in essential physical or chemical reactions at the cell surface. The two organisms studied here in most detail are quite different in many respects. The cells of *V. costicolus* are sticky when grown in all concentrations of salt. On the other hand, the cells of *M. halodenitrificans* are not sticky when grown in salt concentrations of 8 and 16%, or when calcium is added to the medium. The value of calcium in overcoming the deleterious effects of low concentrations of sodium chloride has been observed by Johnson and Harvey (10*a*). The lack of slime in cultures grown in high salt concentrations or in the presence of calcium suggests that the increase in viscosity is linked with the permeability of the cell. It is possible that although the cells appear to grow well in the medium used, the cell membrane lacks certain constituents and may have an altered permeability. Because of its large molecular size, it seems unlikely that DNA itself would diffuse through the membrane. It is possible, however, that the components could diffuse through the membrane and DNA be synthesized, or polymerized, on the outside of the viable cell and retained there. Such an hypothesis may also mean that the DNA, or its components, is either scattered throughout the cytoplasm or even oriented fairly close to the cytoplasmic membrane. When *Achromobacter fischeri*, a luminous marine rod, is placed in hypotonic salt solutions, the nuclear material is stained less readily and appears to be dispersed throughout the cytoplasm (10*b*). A similar dispersal may take place in other halophiles at low salt concentrations and a slight hypotonicity of sodium, calcium, or magnesium may be associated with changes in the cell membrane. A better understanding of the nutrition and cytology of the halophiles will no doubt furnish the information essential to a more complete understanding of the occurrence of extracellular DNA.

Acknowledgment

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STUDIES ON THE EFFECTS OF PHAGOCYTIC STIMULATION ON MICROBIAL DISEASE

IV. THE INFLUENCE OF 1,4-DIMETHYL-7-ISOPROPYLAZULENE WITH AND WITHOUT DIHYDROSTREPTOMYCIN ON EXPERIMENTAL TUBERCULOSIS IN GUINEA PIGS[1]

By László Kátó and Béla Gözsy

Abstract

The outcome of experimental tuberculosis in guinea pigs was favorably influenced by treatment with 1,4-dimethyl-7-isopropylazulene. This compound had no antituberculous properties *in vitro*, but stimulates phagocytic activity of cells of the reticulo-endothelial system. Effects of simultaneous treatment with the azulene derivative and dihydrostreptomycin on well-established tuberculosis were similarly studied. Therapeutical effect of dihydrostreptomycin was significantly increased by the simultaneous treatment with the azulene. This experiment brought evidence that the host–parasite relationship in experimental tuberculosis can be favorably influenced for the benefit of the host by appropriate treatment acting against the parasite and simultaneously stimulating the defense mechanism of the host. The authors discuss the need for more research in tuberculosis therapy directed toward finding ways of stimulating the cell-linked defense mechanism of the host against bacterial invaders.

Introduction

Jancso (6) demonstrated, using perfusion techniques, that antihistamine paralyzed the phagocytic function of the Kupffer cells and that histamine added to the perfusion liquid restored the normal phagocytic function. Jancso (5, 6) has stated that topical histamine applications changed the function of endothelial cells of the small vessels into a phagocytic function; this phenomenon was inhibited by antihistamines. He concluded that histamine is the physiological stimulant for phagocytosis. Biozzi, Mené, and Ovary (1) and Gözsy and Kátó (3) have confirmed Jancso's observations. We reported that certain derivatives of the bicyclo[0,3,5]decapentaene skeleton, of which 1,4-dimethyl-7-isopropylazulene (B.D. I) is the most active, induced phagocytic activity in endothelial cells of small vessels, which normally have no phagocytic property. This derivative, therefore, exhibited the same action as histamine itself. We have also shown (8) that India ink disappeared more quickly from the blood stream of rats pretreated with either histamine or B.D. I. Antihistamines inhibited this stimulated activity of the reticulo-endothelial system (RES). It was found that when antihistamines were administered to rats, intravenously injected India ink disappeared more slowly from the blood stream of the antihistaminized animals than from the controls. Thus, it was inferred that B.D. I might stimulate the RES to an increased activity.

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Contribution from the Institute of Microbiology and Hygiene of the University of Montreal, Montreal, Quebec. This work was partially aided by grants from the Ministry of Health of the Province of Quebec (Federal-Provincial Health Research Grant) and from "Les Fondations Rhéaume".

On the basis of these experiments, it was believed that the host–parasite relationship could be influenced to the benefit of the host in experimentally induced infectious disease. Previous work indicated (4), on the one hand, the deleterious effect of an antihistamine and, on the other hand, the beneficial effects of this RES-stimulating agent on experimental tuberculosis of guinea pigs. B.D. I inhibited the deteriorating effect of the antihistamine (diphenhydramine hydrochloride).

This paper presents experiments that allow the evaluation of B.D. I as an adjunct to the therapy of experimental tuberculosis with a standard antibacterial agent, dihydrostreptomycin, directed towards stimulating the reticulo-endothelial system of the host as well as inhibiting the parasite.

Experimental

Preliminary Experiment

Sixty-two tuberculin-negative guinea pigs, averaging 350–400 gm. in weight, were infected subcutaneously in the left inguinal region with 0.1 mgm. moist weight of tubercle bacilli, Ravenel strain, from a seven-day-old culture on Dubos liquid medium. Twenty days later, six were killed (pretreatment controls) and autopsied. Tuberculous lesions were found in the lungs, liver, and spleen and at the site of inoculation. It may be assumed that the other animals participating in the experiment would show similar lesions of comparable intensity. The remaining 56 animals were divided into seven groups carefully selected as to weight and color, and were treated as follows:

Group 1.—Served as controls. Four animals received a daily subcutaneous injection of 0.5 ml. saline solution. Four others had an intramuscular injection of 0.3 ml. of sesame oil twice weekly.

Groups 2 and 3.—These animals were given twice weekly intramuscular injection of an oily solution of B.D. I, 5.0 mgm., and 25.0 mgm. respectively, in 0.3 ml. sesame oil.

Groups 4 and 5.—These animals were given a daily subcutaneous injection of 5.0 mgm. and 10 mgm., respectively, dihydrostreptomycin (DSM) in 0.5 ml. saline solution.

Group 6.—These animals received 5.0 mgm. DSM daily and 5 mgm. B.D. I twice weekly.

Group 7.—These animals received 5.0 mgm. DSM daily and 25 mgm. B.D. I twice weekly.

The treatment was followed for 55 days except Sundays. The animals were left to survive and at death were autopsied, the pathologic findings were registered, and the cumulative percentage mortality was plotted against time in days of survival after infection.

Previous experiments (4) on the toxicity of B.D. I have shown that doses up to 25.0 mgm. given twice weekly, for 56 days, had no toxic effects and were well tolerated by guinea pigs.

Results

Six pretreatment control animals sacrificed on the 20th day after infection showed macroscopic tuberculous lesions in the lungs, liver, and spleen and at the site of inoculation. Regional lymph nodes were also involved. It was assumed that the 56 animals participating in the experiment had tuberculous lesions of the same importance. At the beginning of treatment, 46 out of 56 animals had open lesions at the site of inoculation.

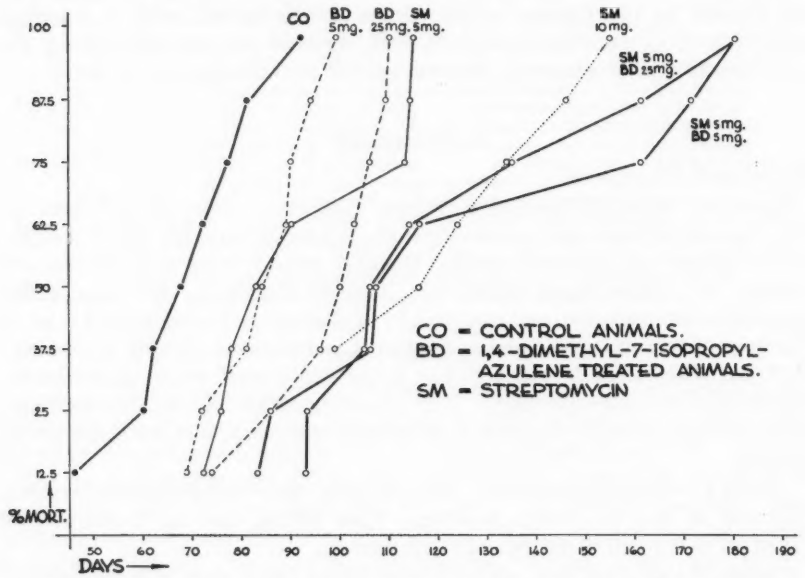


FIG. 1. Cumulative percentage mortality of guinea pigs infected with 0.1 mgm. of tubercle bacilli (Ravenel). Treatment was begun 20 days after infection and continued for 55 days.

Mortality

The percentage mortality for the seven groups is shown in Fig. 1. The massive doses of a highly virulent strain of *Mycobacterium tuberculosis* (Ravenel) killed all control animals within 93 days. The percentage mortality was as follows:

At 93 days: 100% of the controls,
62.5% of those treated with 5 mgm. of DSM,
25% of those treated with 25 mgm. of B.D. I;

At 115 days: 100% of those treated with 5 mgm. of DSM,
62% of those treated with 5 mgm. of DSM plus 5 mgm. B.D. I,
50% of those treated with 5 mgm. of DSM plus 25 mgm. B.D. I.

The percentage mortality for the animals treated with 5 mgm. of DSM plus either 5 mgm. or 25 mgm. of B.D. I was practically the same as for those treated with 10 mgm. of DSM.

Findings at Autopsy

In this experiment, the antituberculous activity of the given substance (B.D. I) was based on percentage mortality. Thus, pathologic findings at autopsy are less significant and will be studied in the main experiment. It was surprising to note that the spleens and lungs were markedly less involved in animals treated with B.D. I or DSM plus B.D. I than they were in control or DSM-treated animals. The inguinal lesions of B.D. I treated animals showed a relatively rapid tendency to heal.

Main Experiment

One hundred and seventy-eight tuberculin-negative guinea pigs averaging 400 gm. were infected in the inguinal region with 0.1 mgm. moist weight of tubercle bacilli (strain H37Rv) from a seven-day-old culture in Dubos liquid medium. Twenty days later 10 were killed (pretreatment controls) and autopsied. The remaining 168 animals were divided into 14 groups according to color and weight so that two sets of seven groups could be submitted to experiment. The corresponding groups of animals in each of the sets, 1–7 and I–VII, received identical treatment as follows:

Group 1.—Served as controls. Six were given 0.5 ml. of saline solution daily. The other six received 0.3 ml. sesame oil (intramuscularly (i. m.)) twice weekly.

Group 2.—These animals received 0.5 mgm. of B.D. I in 0.3 ml. sesame oil i.m. twice weekly.

Group 3.—These were treated with 10 mgm. of B.D. I in 0.3 ml. sesame oil i.m. twice weekly.

Group 4.—These received 5 mgm. of DSM daily.

Group 5.—These received 5 mgm. of DSM daily and 10 mgm. of B.D. I twice weekly.

Group 6.—These were given 15 mgm. of DSM daily.

Group 7.—These received 15 mgm. of DSM daily and 10 mgm. of B.D. I twice weekly.

The treatments continued for 56 days, except Sundays. The surviving animals of groups 1 to 7 were killed at 88 days after infection, which was 12 days after treatment was ended. The animals were autopsied and macroscopic lesions registered. The lungs, liver, spleen, and lymph nodes were submitted to histologic examinations. The animals of groups I to VII were allowed to survive and, at death, were autopsied, the pathologic findings were recorded, and the cumulative percentage mortality was plotted against time in days of survival after infection. The animals were weighed weekly.

Results

Ten pretreatment control animals killed at 20 days after infection showed grossly visible tuberculous lesions in the lungs, liver, and spleen and at the site of inoculation. The regional lymph nodes were involved. It was assumed that the 168 animals participating in the experiment would show tuberculous lesions of similar importance. All remaining animals, at that time, either presented large nodules or showed ulcerations and skin perforation at the site of inoculation.

Observations on Groups 1–7

Mortality.—In the untreated group, at 88 days after infection, four animals had died and showed large tuberculous lesions. Two animals had died among those treated with 0.5 mgm. of B.D. I and three of those receiving 10 mgm. of B.D. I. These last three animals showed no visible macroscopic tuberculous lesions but had enlarged inguinal nodes. All three died between the 76th and 79th day after infection. The cause of death could not be determined, but it was certainly due to technical difficulties in the intramuscular administration of the oily solution. At necropsy, some blue oil from B.D. I was found in the large pulmonary vessels. All other animals were alive at 88 days.

Change in weight.—The animals still alive at the end of the experiment had gained weight. The control animals, those given 0.5 mgm. B.D. I, and those receiving DSM had gained an average of 85–110 gm. and the surviving animals in the other groups had gained 140–185 gm., which indicated that the amount of drug was well tolerated.

Visible findings at necropsy.—Fig. 2 gives as chematic presentation of the extent of tuberculous lesions in each animal at necropsy. The organs which showed severe diffuse involvement are completely blackened. Miliary and nodular lesions are presented by smaller and larger dots. An arrow on the right inguinal site means an ulcer at the site of infection. Dots at inguinal, sacral, or axillary sites mean involvement of lymph nodes. The representation of the extent of the disease was slightly modified from that described by Karlson and Feldman (7). An extensive tuberculous involvement of the sites of predilection was seen in the untreated control animals as well as in animals treated with 5 mgm. of DSM only, except in two of these treated animals. Six animals in the group receiving 0.5 mgm. of B.D. I and six animals of the group receiving 10 mgm. of B.D. I had no visible lesions in the lungs, liver, and spleen. Likewise, six animals in the group treated with 5 mgm. of DSM and 10 mgm. of B.D. I had no visible lesions in the lungs, liver, and spleen, but it was surprising to find no macroscopic lesions in the lungs of any of the 12 animals in Group 5. No significant difference was found at necropsy between the animals treated with 15 mgm. of DSM with and without B.D. I. Histopathologic differences between these two groups will be discussed below.

Histopathology

Pretreatment control animals had multiple active tubercles in the lungs, spleen, and liver, that were progressive and even necrotic in a few animals.

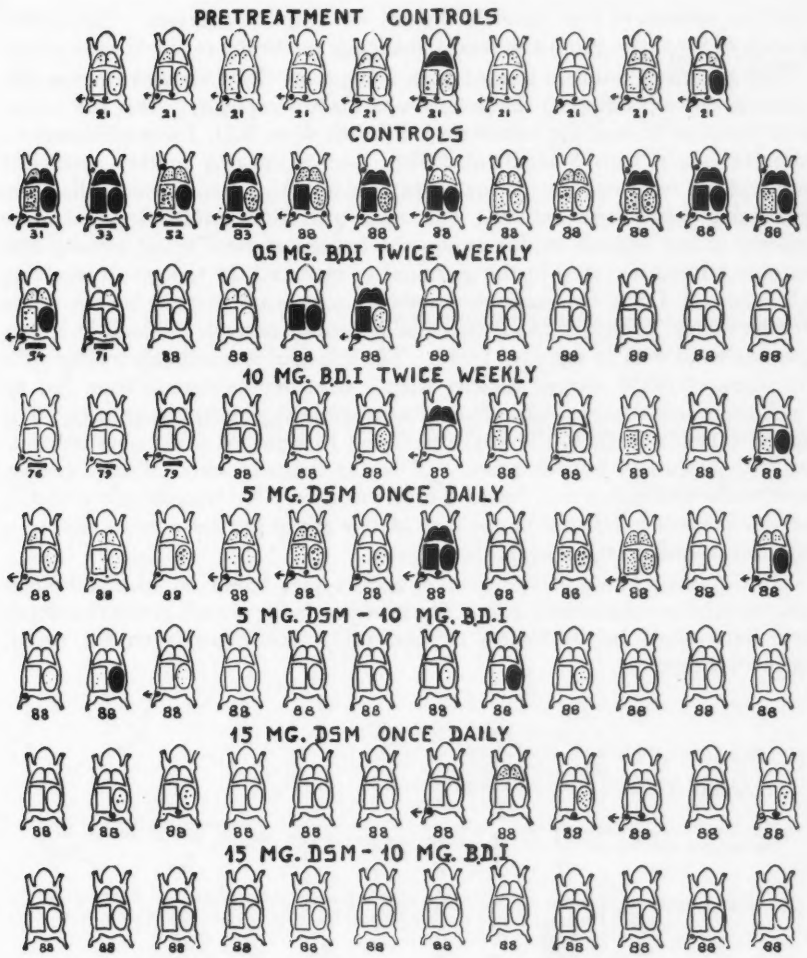


FIG. 2. Schematic presentation of the extent of tuberculous lesions at necropsy in the first experiment. Treatment was begun at 20 days after infection. All surviving animals were killed at 88 days, which was 12 days after treatment was ended. The day of autopsy is given under each animal. See text for explanation.

It is assumed that, when treatment was begun, all other animals had histopathologic lesions comparable in extent and character. Of the six animals treated with 0.5 mgm. of B.D. I which showed no visible lesions at necropsy, three were found, however, to present microscopic calcified foci in the spleens and two had small but active tuberculous lesions in the liver. The animals in the group receiving 10 mgm. of B.D. I showed no visible lesions, though some miliary epitheloid infiltrations were found in the lungs of two animals,

and the spleens of four animals showed an incipient necrosis. The lymph glands of these two groups showed a tendency to fibrotic repair in most cases.

The organs of animals treated with 5 mgm. of DSM showed microscopic pictures corresponding to the lesions seen macroscropically. But the extent and characteristics of the lesions were different when B.D. I was administered simultaneously with 5 mgm. of DSM. Lungs showing miliary epitheloid infiltrations were densely packed with monocytes and epitheloid cells. No coalescing parts were detected but more interstitial infiltrations. In the spleens of the animals of this group, the follicles showed a fair activity and intense mitosis occurred in the germinative centers. In the group receiving 15 mgm. of DSM five animals presented coalescing necrosis in the sacral lymph nodes. None of the lymph nodes became necrotic when B.D. I was administered with 15 mgm. of DSM. The spleen of four animals treated with 15 mgm. of DSM showed follicles with a tendency to degeneration but no such thing was observed when B.D. I was administered simultaneously. The lungs of two animals in the group receiving 15 mgm. of DSM plus 10 mgm. of B.D. I showed fibrotic scars, and in the lungs of two animals, miliary epitheloid infiltration was found but no necrosis. No microscopic evidence of tuberculosis was found in the liver of this group but the spleens showed a marked mitosis in the germinative centers.

The average index of infection in guinea pigs based on histopathologic characteristics appears in Fig. 3. The infection index of each group of animals was determined by Feldman's method (2), which depends on the extent of involvement.

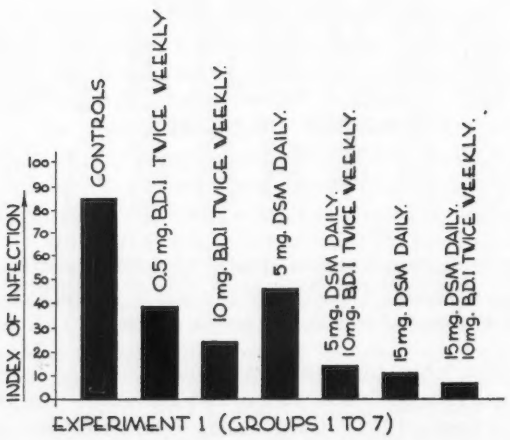
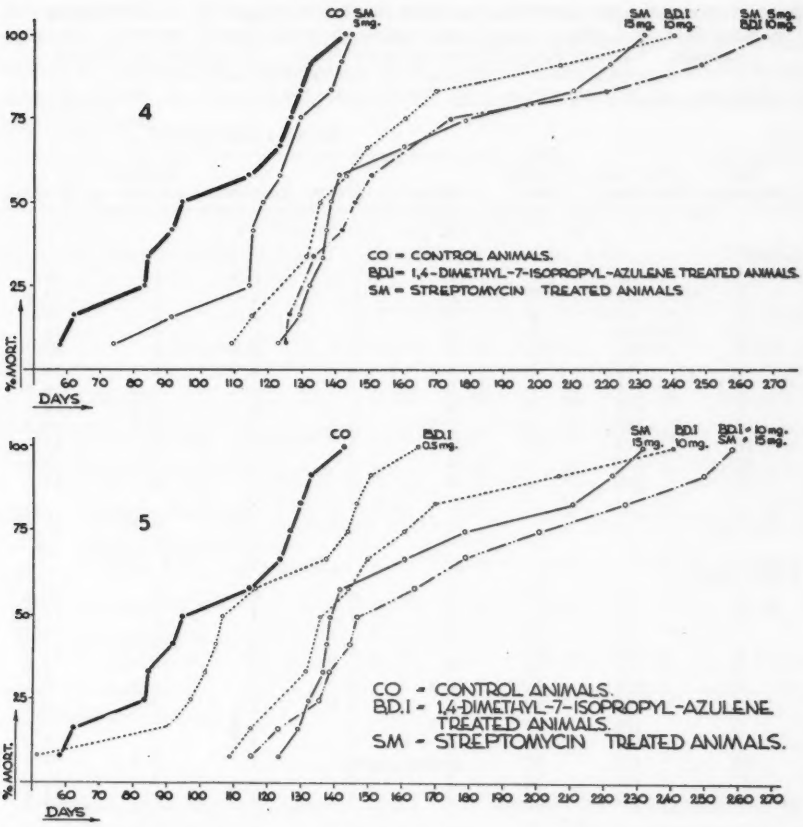


FIG. 3. Average index of infection in guinea pigs of the first experiment, based on histopathologic characteristics. Values of 10 to 35 were given for organs with actively progressing tuberculous lesions. Values of 1 to 3 were given for healed lesions and no necrosis. Treatment was started on the 20th day of infection. Surviving animals were killed at the 88th day, which was 12 days after treatment was ended.



FIGS. 4 and 5. Mortality rate of guinea pigs participating in the second experiment. Treatment was begun 20 days after infection and was continued for 56 days.

Observations on Groups I to VII

In this experiment, the survival time of animals was studied and visible lesions were recorded at necropsy. Figs. 4 and 5 show the mortality rates for the animals participating in the study.

In Table I, the results of treatment with 5 mgm. of DSM daily and 5 mgm. of DSM daily plus 10 mgm. of B.D. I twice weekly are compared with control animals, using the visible lesions as the index of disease. Each organ was given a rating of 0 to 4 according to the amount of gross tuberculous disease.

TABLE I

RESULTS OF TREATMENT WITH 5 MGM. OF DSM DAILY AND WITH 5 MGM. OF DSM DAILY PLUS 10 MGM. OF B.D. I TWICE WEEKLY ARE COMPARED WITH VISIBLE LESIONS OF CONTROL ANIMALS. THERAPY BEGUN 20 DAYS AFTER INFECTION. THE EXTENT OF LESIONS WAS REGISTERED AT AUTOPSY ON THE DAY THE ANIMALS DIED

Guinea pigs number	Survival in days	Days of treatment	Index of tuberculosis*				
			Lungs	Liver	Spleen	Lymph nodes	Totals
Control							
1	58	0	4	2	1.5	3	10.5
2	62	0	2	4	4	3.5	13.5
3	83	0	2	2	4	2	10.0
4	84	0	4	4	4	4	16.0
5	91	0	4	2.5	2	4	12.5
6	94	0	4	4	4	4	16.0
7	114	0	4	4	4	2.5	14.5
8	123	0	4	4	4	2.5	14.5
9	126	0	1.5	2.5	4	2.5	10.5
10	129	0	1.5	4	4	4	13.5
11	132	0	4	4	4	3	15.0
12	142	0	4	4	4	2	14.0
Averages			3.25	3.42	3.62	3.08	13.37
DSM 5 mgm.							
1	74	54	2	4	4	1	11.0
2	91	56	4	3.5	4	1	12.5
3	114	56	2	3.5	4	2.5	12.0
4	115	56	3	4	4	3	14.0
5	115	56	0	0.5	1	2	3.5
6	118	56	2.5	1	1	1.5	6.0
7	123	56	0	1.5	1.5	2	5.0
8	129	56	1.5	0	1.5	2	5.0
9	129	56	0	0	1.5	1.5	3.0
10	138	56	3	1	1	2.5	7.5
11	141	56	1	4	4	4	13.0
12	144	56	2.5	4	4	4	14.5
Averages			1.79	2.25	2.63	2.25	8.92
DSM 5 mgm., B.D. I 10 mgm.							
1	125	56	0	0	0	1	1.0
2	126	56	0	0	1	1	2.0
3	133	56	0	0	0	0.5	0.5
4	133	56	0	0	0	0.5	0.5
5	142	56	4	0	1	1	6.0
6	145	56	0.5	1	1	0.5	3.0
7	150	56	0	1.5	0	0.5	2.0
8	173	56	0	2	0.5	1	3.5
9	173	56	0	1.5	2.5	2	6.0
10	220	56	1.5	1.5	2	1	6.0
11	248	56	1.5	2	1.5	1	6.0
12	267	56	1.5	1.5	1.5	1.5	6.0
Averages			0.75	0.91	0.91	0.95	3.52

* *Each organ was given a value of 0 to 4 according to the amount of gross tuberculous disease.*

The rates of survival in the group receiving 15 mgm. of DSM alone and in the group where 15 mgm. of DSM was administered simultaneously with 10 mgm. of B.D. I did not differ, but there was a difference between the lesions in the animals of the two groups as seen at autopsy. Therefore, we give, in Table II, the tuberculosis index of the organs for the animals of this experiment.

TABLE II

RESULTS OF TREATMENT IN THE SECOND EXPERIMENT. THERAPY BEGUN 20 DAYS AFTER INFECTION. THE EXTENT OF TUBERCULOUS LESIONS WAS REGISTERED AT AUTOPSY ON THE DAY THE ANIMALS DIED

Group	Treatment	Average index of tuberculosis*				
		Lungs	Liver	Spleen	Lymph nodes	Totals
I	Controls	3.25	3.42	3.62	3.08	13.37
II	0.5 mgm. B.D. I twice weekly	2.84	2.86	3.02	3.02	11.74
III	10 mgm. B.D. I twice weekly	1.11	1.45	1.86	1.75	6.17
IV	5 mgm. DSM daily	1.79	2.25	2.63	2.25	8.92
V	5 mgm. DSM daily and 10 mgm. B.D. I twice weekly	0.75	0.91	0.91	0.95	3.52
VI	15 mgm. DSM daily	1.16	1.22	1.68	1.84	5.90
VII	15 mgm. DSM daily and 10 mgm. B.D. I twice weekly	0.62	1.12	0.50	0.91	3.15

* *Value as in Table I.*

Discussion

1,4-Dimethyl-7-isopropylazulene (B.D. I) showed no bacteriostatic or bactericidal activity, *in vitro*, against tubercle bacilli. Earlier experiments in this laboratory have demonstrated, however, that B.D. I increases the phagocytic activity of the RES. We have produced evidence that this non-toxic agent has beneficial effects on experimental tuberculosis of guinea pigs (4) and when B.D. I is administered simultaneously with DSM the efficacy of DSM could be significantly increased. The drug also inhibited the deleterious effects of antihistaminic substances on experimental tuberculosis of guinea pigs (4). The tendency in research directed toward the treatment of tuberculosis is to look for more effective specific chemotherapeutic or antibiotic agents. Meanwhile, little has been done to stimulate the host in its fight against invading bacteria. On the basis of the interesting properties of B.D. I we suggest the development of a new field in experimental tuberculosis: an attempt to attack the parasite by the use of a drug acting on the tubercle bacillus and simultaneously to stimulate the cell-linked defense mechanism of the host.

An intramuscular application of 25 mgm. of B.D. I twice weekly prolonged the lives of infected animals as much as did 5 mgm. of DSM given daily. Furthermore daily 5 mgm. doses of DSM administered simultaneously with doses of 5 mgm. or 25 mgm. of B.D. I twice weekly have proved to be as efficient as daily 10 mgm. doses of DSM.

By the end of 93 days, 100% of the untreated animals and 62.5% of the 5 mgm. DSM treated animals had died, whereas 25% of those which had received 5 mgm. DSM plus 25 mgm. B.D. I had died.

In the preliminary experiment, the extent of lesions found in controls showed that a widespread progressive tuberculosis had been established before treatment was started. We wish to point out this fact, because it shows evidence of the curative value of a given substance or combination of substances.

In regard to the main experiments, it should be pointed out that the highly virulent strain of tubercle bacilli had produced a well-established progressive tuberculosis in the guinea pigs at the time the treatment was begun. The first experiment brought evidence that B.D. I alone, in relatively small doses (0.5 mgm. and 10 mgm.) given twice weekly, influenced favorably the course of the disease. From the second experiment it can be concluded again that B.D. I lengthens considerably the survival time of tuberculous animals when it is administered alone. The efficacy of 5 mgm. of DSM daily (a suboptimal dose) is significantly higher when the cell-linked defense mechanism of the host is stimulated simultaneously by means of B.D. I. The survival rate of animals did not differ between the groups when 15 mgm. of DSM and 15 mgm. of DSM plus 10 mgm. of B.D. I were administered but visible lesions were less in the latter group. These findings were due to the facts that treatment was abandoned after 56 days, and that effective doses of DSM (15 mgm.) have been used. It is an accepted principle that when an experimental study is made of a combination of two or more chemotherapeutic agents, the dose of each drug must be sufficiently small that one of the drugs alone could not be responsible for the therapeutic effects observed. In this study we deviated from this principle because one of the agents used was not an antibacterial one.

We conclude from our previous studies (4) and from the present one that certain well-defined substances have considerable therapeutic effect on experimental tuberculosis without exhibiting any antibacterial properties. We believe that histamine plays an important part in this stimulation of the RES. If, however, more information could be uncovered as to the mechanism by which histamine acts in this respect, this would point the way to further searches for substances acting by such a mechanism and for substances which might have still greater effectivenes in the treatment of tuberculosis. This new approach to the problem may be particularly desirable because of the slow but constant development of resistance to chemotherapeutic agents on the part of the tubercle bacillus.

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THE EFFECT OF PENICILLIN ON CO_2 PRODUCTION AND THE UPTAKE OF C^{14}-LABELLED GLUCOSE AND GLUTAMINE BY *STAPHYLOCOCCUS AUREUS*[1]

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Abstract

Radioactive glucose or glutamine was supplied to growing and non-growing cultures of *Staphylococcus aureus* 6538 in the presence of various concentrations of penicillin. Total CO_2 and $C^{14}O_2$ production were measured, and the uptake and distribution of carbon from glucose and glutamine in both growing and non-growing cultures were determined. The production of CO_2 from glucose was stimulated by penicillin, that from glutamine was suppressed, while total CO_2 production was unaffected. Neither glucose nor glutamine were major substrates of respiration. Penicillin exerted widely different effects on the uptake and incorporation of carbon from both glucose and glutamine in growing and non-growing cells. From a consideration of these effects, it is suggested that penicillin affects cells at two or more specific points in their metabolism. It was noted that CO_2 production continued at a high rate in non-growing cultures although cell division had virtually ceased.

Introduction

Although several investigators have examined the mechanism of penicillin action (5, 6) and resistance (3, 4), little is yet known of the nature of penicillin effect on bacteria or its site of operation in the cell. In an effort to clarify the situation, a study has been initiated to examine the effect of penicillin on the metabolism of various compounds in growing and non-growing bacterial cells. The present communication deals with the effect of penicillin on some aspects of the metabolism of glucose and glutamine by *Staphylococcus aureus* 6538, a highly susceptible strain.

Materials and Methods

Cultures

Stock cultures of *S. aureus* 6538 were kept at 0°C. in Robertson's meat medium. One 3 mm. loop of stock culture was added to 5 ml. of heart infusion broth and incubated for 17 hr. at 37.5° C. to provide a standard inoculum. Experimental cultures were provided by adding 1.0 ml. of standard inoculum to each of eight 500 ml. Florence flasks containing 250 ml. of heart infusion broth. The experimental heart infusion broth cultures were grown at 37.5° C. until shortly before needed (four hours for growing cultures, 11 hr. for non-growing cultures) when samples were withdrawn from the flasks and cell densities determined.

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Cell Density Measurements

For the production of growth curves, experimental cultures were produced as outlined above, and 40 ml. samples were taken at the appropriate time intervals. The cells were centrifuged from the 40 ml. samples at once and resuspended in 10 ml. of distilled water. The density of this suspension was immediately read against distilled water in a Coleman Jr. spectrophotometer at 605 mμ, using matched culture tubes, 14 mm. internal diameter, as cuvettes. In order to check the densities of subsequent experimental cultures, cells were rapidly centrifuged from 5 ml. samples, resuspended in distilled water, and read as before. Only those flasks whose cell densities agreed within one per cent of a previously established norm were used. Four of the eight flasks were chosen for each series of experiments, the rest being discarded.

Experimental Procedure

When the experimental heart infusion broth cultures had been incubated for 4½ hr. (growing cultures) or 11½ hr. (cultures had stopped growing), 1.0 ml. of penicillin-G solution was added to each flask to give concentrations of penicillin of 0, 0.02, 0.5, and 10.0 International Units per milliliter of culture. The flasks were incubated a further half hour, when 10.0 ml. of a solution containing 45.0 mgm. of glucose, and 6.0 mgm. of glutamine per 10 ml. were added to each flask. Either the glucose or the glutamine, synthesized as described elsewhere (1, 7), contained three to four microcuries of equally distributed C^{14} label. The cultures were incubated for a further period of two hours during which a stream of CO_2-free air was passed through the air space over the culture. This constituted the experimental period. All cultures were examined at the end of the experimental period for signs of contamination by other microorganisms, and were found free of contamination. The quantities of both glucose and glutamine fed were approximately 20 times the amounts which actively growing cells would take up during the experimental period. It was found in preliminary trials that these amounts were the lowest that would allow the compounds to be taken up at maximum rate.

Determination of CO_2 Production

Evolved CO_2 was trapped in NaOH during the two-hour experimental period, after which the cultures were acidified with 1*N* H_2SO_4 and boiled briefly to drive off CO_2 bound in the medium, which was also trapped. Total evolved CO_2 was measured titrimetrically and precipitated as $BaCO_3$. The $BaCO_3$ was washed three times with distilled water, suspended in a small volume of distilled water, and plated on aluminum planchets. Their radioactivity was determined in a gas-flow counter, and the quantity of $BaCO_3$ plated was determined by weighing. From these data, using appropriate correction factors to allow for self absorption, coincidence, backscattering, and the characteristics of the counter, the total and specific activities of carbon in the CO_2 were determined (7). Since the heart infusion broth contained no appreciable quantities of either glucose or glutamine, the specific

activity of carbon in the fed compounds remained unchanged after their addition to the medium. The fraction of total evolved CO_2 derived from the radioactive compound offered was determined by dividing the specific activity of carbon in the evolved CO_2 by the specific activity of carbon in the fed radioactive compound. From this and the total amount of evolved CO_2 it was possible to determine the absolute amount of CO_2 derived from the added radioactive compound.

Determination of C^{14} Uptake and Distribution

Cultures used for CO_2 determination could not be used for uptake measurements because of the acid treatment. Cultures were grown and treated as above, except that at the end of the two-hour experimental period the cells were centrifuged from the medium, washed twice with distilled water, and extracted twice with boiling 80% ethanol. After removal of the residues the extracts were acidified slightly with HCl to remove CO_2. The alcohol insoluble residues were treated with 3*N* HCl at 100° C. for three hours to dissolve them. Extracts and residues were evaporated to dryness *in vacuo* and redissolved in water. Aliquots of these solutions were plated on aluminum planchets and their radioactivity was determined (7). Total uptake of activity was determined by adding the values obtained for alcohol soluble and insoluble, and respired activities.

Expression of Results

It has been assumed that the densities of the growing cultures were directly proportional to the numbers of actively metabolizing cells they contained, since they were in the logarithmic phase of growth. It has been observed that culture densities parallel closely the viable cell counts of both growing and non-growing cultures (8). Providing that the viable cell count is proportional to the number of metabolizing cells in non-growing cultures, it may be assumed that the densities of non-growing cultures were also in proportion to the number of metabolizing cells they contained. Since the densities of non-growing cultures varied little in these experiments, errors in these assumptions will have little if any effect on the interpretation of data from such cultures. In cultures where the density was decreasing during the experiment, density readings probably gave a rather high proportional value for the number of metabolizing cells because of the presence of dead cells which had not disintegrated.

Average densities of the cultures during the two-hour experimental period were calculated from the curves shown in Fig. 1, and are given in Table I, together with the per cent increase in density of the cultures during the experimental period. Total respired CO_2 and the $C^{14}O_2$ produced from glucose and glutamine have been divided by the average culture densities to give relative values for the production of CO_2 per unit culture density, and hence per cell. These results are shown in Tables I and II. The data for the uptake and distribution of radioactive carbon from the fed compounds, shown in

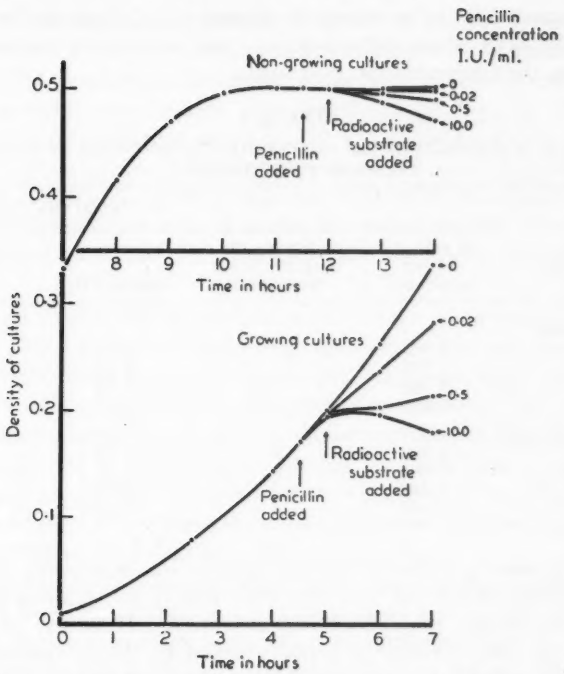

FIG. 1. The growth of *Staphylococcus aureus* 6538 cultures in the presence of varying concentrations of penicillin.

Tables III and IV, have been calculated by dividing the activities in various fractions by the specific activity of the fed carbon. In order to show more clearly the effects of penicillin on the distribution of active carbon in cells, the data in Tables III and IV have been divided by the average culture densities and expressed as percentages of the values obtained for the growing cultures lacking penicillin. These results are shown in Tables V and VI.

The activity data from which the results were calculated have a probable error of 2% for CO_2 measurements, 4% for the soluble and insoluble activities of growing cultures, and 8% for the soluble and insoluble activities of non-growing cultures.

Results and Discussion

Cultures produced in the manner outlined above grew rapidly with little or no lag phase, as shown in Fig. 1. The cultures had reached maximum density after 11 hr., following which no further increase was observed. In separate experiments it was shown that culture density remained constant for as long as eight hours after the maximum had been reached, before it began to decrease.

Penicillin caused little or no change in growth rate during the first half hour after addition, after which culture density was reduced by varying amounts depending on the concentration of penicillin.

TABLE I

THE EFFECT OF PENICILLIN ON THE GROWTH AND CO_2 PRODUCTION OF *Staphylococcus aureus* 6538 CULTURES

Penicillin concentration, I.U./ml.	Average density of culture during experiment	% increase in density of culture during experiment	Total CO_2 produced	
			Mgm. CO_2	Mgm. CO_2 / Density
Growing cultures				
0	0.265	68	6.81	25.7
0.02	0.239	41	6.13	25.6
0.5	0.203	7.5	5.51	27.1
10.0	0.193	−7.5	4.43	23.0
Non-growing cultures				
0	0.502	1	9.34	18.6
0.02	0.500	0	9.32	18.6
0.5	0.495	−2	9.34	18.8
10.0	0.487	−6	7.97	16.4

It may be seen in Table I that CO_2 production of growing cultures was decreased by the addition of penicillin while that of non-growing cultures was unaffected except by the highest concentration. However, in both, CO_2 production per unit density was not affected by lower concentrations of penicillin and only slightly by the highest, where the contribution of dead cells to the density may have caused the discrepancy. It appears that while penicillin slowed or stopped the multiplication of cells in these cultures it did not affect the rate of CO_2 production of individual cells. It is of interest to note that CO_2 production per unit density of non-growing cultures was relatively great, approximately three-quarters that of actively growing cultures.

TABLE II

THE EFFECT OF PENICILLIN ON CO_2 PRODUCTION FROM C^{14}-LABELLED GLUCOSE AND GLUTAMINE BY *Staphylococcus aureus* 6538

Penicillin concentration, I.U./ml.	CO_2 produced from glucose			CO_2 produced from glutamine		
	% total CO_2	μgm. CO_2	μgm. CO_2 / Density	% total CO_2	μgm. CO_2	μgm. CO_2 / Density
Growing cultures						
0	4.0	272	1020	0.095	6.47	24.4
0.02	4.4	270	1130	0.093	5.70	23.8
0.5	4.8	264	1300	0.037	2.04	10.0
10.0	5.2	231	1190	0.027	1.20	6.2
Non-growing cultures						
0	4.0	374	745	0.11	10.3	20.5
0.02	4.1	382	764	0.10	9.32	18.6
0.5	4.5	421	850	0.043	4.01	8.1
10.0	4.7	375	770	0.012	0.96	2.0

Table II shows that the production of CO_2 from glucose by both growing and non-growing cultures without penicillin accounted for about four per cent of the total CO_2 production, increasing steadily to about five per cent with increasing penicillin concentration. On the other hand, the production of CO_2 from glutamine accounted for only about 0.1% of the total CO_2 production, and was strongly suppressed by increasing concentrations of penicillin, more so in non-growing than in growing cultures. CO_2 production from glucose per unit density was increased as much as 25% by penicillin, while from glutamine it was decreased to $\frac{1}{4}$ and 1/10th respectively in growing and non-growing cultures.

The over-all picture for respiration is not clear. Approximately 96% of the respired CO_2 was derived from compounds other than glucose and glutamine, presumably from the nutrient medium. However, since the total CO_2 production of cells is unaffected by penicillin, and since the changes observed in CO_2 production from glucose and glutamine constitute a very small part of total respiration, it is reasonable to assume that the major substrates of respiration are unaffected. It is difficult to believe that large increases and decreases could have taken place in major substrates, balancing each other so precisely as to leave the total respiration unchanged.

The uptake and distribution of carbon from glucose and glutamine is shown in Tables III and IV. It may be seen that in both growing and non-growing cultures the distribution of glucose carbon was different from that of glutamine, and that the distribution of carbon from the fed compounds in growing cultures was very different from that in non-growing cultures. Of particular interest is the small amount of glucose and glutamine carbon incorporated into the alcohol-insoluble residues of non-growing cultures.

TABLE III

THE EFFECT OF PENICILLIN ON THE UPTAKE AND DISTRIBUTION OF CARBON FROM C^{14}-LABELLED GLUCOSE BY *Staphylococcus aureus* 6538 CULTURES, AS MICROGRAMS OF CARBON PER CULTURE

Penicillin concentration, I.U./ml.	Respired carbon	Alcohol soluble carbon	Alcohol insoluble carbon	Total uptake of carbon
Growing cultures				
0	74.1	166	610	850
0.02	73.6	144	421	639
0.5	71.8	86	142	300
10.0	63.0	86	76	225
Non-growing cultures				
0	102	2.0	0.41	105
0.02	104	2.0	0.43	109
0.5	115	1.8	0.41	119
10.0	102	1.4	0.40	106

TABLE IV

THE EFFECT OF PENICILLIN ON THE UPTAKE AND DISTRIBUTION OF CARBON FROM C^{14}-LABELLED GLUTAMINE BY *Staphylococcus aureus* 6538 CULTURES, AS MICROGRAMS OF CARBON PER CULTURE

Penicillin concentration, I.U./ml.	Respired carbon	Alcohol soluble carbon	Alcohol insoluble carbon	Total uptake of carbon
Growing cultures				
0	1.765	60.3	78.9	141.0
0.02	1.555	39.4	53.8	94.8
0.5	0.558	10.8	30.4	41.8
10.0	0.328	8.3	20.4	29.0
Non-growing cultures				
0	2.81	0.25	0.047	3.11
0.02	2.54	0.23	0.045	2.81
0.5	1.09	0.23	0.045	1.36
10.0	0.265	0.18	0.035	0.48

The effects of penicillin on the uptake and distribution of carbon from glucose and glutamine by individual cells (i.e. on a density basis) are shown more clearly in Tables V and VI. Penicillin had little or no effect on the incorporation of active carbon into alcohol soluble or insoluble fractions of non-growing cultures, but caused a marked decrease in growing cultures. In growing cells the incorporation of carbon from glutamine into the alcohol soluble fraction was more markedly suppressed than from glucose, but the reverse was true for the incorporation of carbon into the insoluble residues. The effect of penicillin on total uptake is variable, since it is a composite of its effects on various fractions.

TABLE V

THE EFFECT OF PENICILLIN ON THE UPTAKE AND DISTRIBUTION OF CARBON FROM C^{14}-LABELLED GLUCOSE BY *Staphylococcus aureus* 6538 CELLS

Data from Table III divided by culture densities and expressed as per cent of the growing culture lacking penicillin

Penicillin concentration, I.U./ml.	Respired carbon	Alcohol soluble carbon	Alcohol insoluble carbon	Total uptake of carbon
Growing cells				
0	100	100	100	100
0.02	110	96	77	83
0.5	126	68	30	46
10.0	117	71	17	36
Non-growing cells				
0	73	0.63	0.035	6.5
0.02	74	0.64	0.037	6.8
0.5	83	0.58	0.036	7.5
10.0	75	0.47	0.036	6.8

TABLE VI

THE EFFECT OF PENICILLIN ON THE UPTAKE AND DISTRIBUTION OF CARBON FROM C^{14}-LABELLED GLUTAMINE BY *Staphylococcus aureus* 6538 CELLS

Data from Table IV divided by culture densities and expressed as per cent of the growing culture lacking penicillin

Penicillin concentration, I.U./ml.	Respired carbon	Alcohol soluble carbon	Alcohol insoluble carbon	Total uptake of carbon
Growing cells				
0	100	100	100	100
0.02	98	72	76	75
0.5	41	23	50	39
10.0	25	19	35	28
Non-growing cells				
0	84	0.22	0.031	1.2
0.02	76	0.20	0.030	1.1
0.5	33	0.20	0.030	0.5
10.0	8	0.16	0.024	0.2

Penicillin had widely different effects on the evolution of radioactive CO_2 on one hand and on the incorporation of radioactive carbon into soluble and insoluble fractions on the other. CO_2 production from both glucose and glutamine was affected similarly in both growing and non-growing cultures, while incorporation was affected only in growing cultures. The latter may be due to the fact that growing cells must necessarily be carrying out many reactions which are absent from non-growing cells. It appears that the action of penicillin is at least twofold, affecting both synthetic and catabolic types of reactions. Since penicillin affected neither the incorporation of active carbon by non-growing cells, nor the total CO_2 production of growing and non-growing cells (Table I), it is concluded that its action is confined to a few specific reactions.

At first glance the present data appear to support the view that penicillin prevents the uptake of metabolites, thus causing the cell to die for the lack of certain essential nutrients (4). However, closer inspection reveals certain inconsistencies with this view. Firstly, it may be seen from Table V that the uptake of glucose carbon by non-growing cells was not suppressed by penicillin. Secondly, although the uptake of glucose by growing cells was suppressed by penicillin, CO_2 production from glucose was increased. Hence the amount of glucose taken up could not have been limiting, even in the presence of the highest concentration of penicillin. Thirdly, it may be seen from Table IV that the uptake of glutamine carbon in the growing culture in the presence of the highest concentration of penicillin was nearly ten times as great as in the non-growing, untreated culture, yet CO_2 production from glutamine by the growing culture was very much depressed. The very low uptake of glutamine carbon by the non-growing, untreated culture was sufficient to support a relatively high level of CO_2 production from glutamine.

Hence it does not appear that the reduction of CO_2 production from glutamine in the growing cultures was the result of a limiting amount of glutamine entering the cells. Finally, it may be seen that although the total uptake of glutamine carbon by non-growing cultures was suppressed by penicillin, the amounts incorporated into the alcohol soluble and insoluble fractions of the cells were little affected. In view of all these points it does not appear likely that the observed decreases in respiration and incorporation in the presence of penicillin were the results of the inability of the fed compounds to enter the cell. It is more likely that decreased uptake was the result of some effect of penicillin on the metabolism in the cell, and hence the utilization of the compounds.

The theory that penicillin increases the rate of oxidation reactions, causing the cell to "burn itself out" in a short time (6), is not supported by the present results. If oxidation does proceed at high speed, the oxidation of carbon containing substrates must be incomplete, since CO_2 production is unaffected by penicillin. Though the rate of oxidation of glucose is somewhat enhanced by penicillin, this substrate accounts for only 4% of respired CO_2. On the other hand, the oxidation of glutamine to CO_2 is strongly suppressed by penicillin.

Apart from penicillin effects, two points of interest arise from the data. The first is the low absolute level of CO_2 production from glutamine by *S. aureus* 6538. Other strains of *S. aureus* have also been shown in separate experiments to produce CO_2 from glutamine at a very low rate. Plants have been shown to oxidize glutamine to CO_2 via the Krebs cycle at a relatively high rate (2). It appears possible either that the oxidative metabolism of *S. aureus* does not proceed via the Krebs cycle or that glutamine is unable to enter such a cycle in this organism.

The second point is the strikingly small amounts of active carbon incorporated into the alcohol soluble and insoluble portions of the cells in non-growing cultures when radioactive glucose or glutamine was fed, as shown in Tables III and IV. The growing cultures incorporated over one thousand times as much carbon from both glucose and glutamine into their alcohol insoluble residues as did similar non-growing cultures. Even the growing cultures whose growth had been completely stopped by high concentrations of penicillin incorporated from two to four hundred times as much active carbon into their insoluble residues as did non-growing cultures. It may be seen in Tables V and VI that growing cells incorporated approximately three thousand times more active carbon into their insoluble residues than did non-growing cells. However, as is shown in Table I, CO_2 production of the cells in non-growing cultures continued at three-quarters of the rate of those in the logarithmic phase of growth. Similar experiments have shown that non-growing cultures of *S. aureus* 6538 and D_2, whose densities, total cell counts, and viable cell counts remained approximately constant during 18 hr. incubation at 37° C., incorporated into their cell residues less than 0.004%

of the activity offered to them as either glucose or glutamine. During this time CO_2 production proceeded at about the same rate as in the non-growing cultures used in the present experiments.

Non-growing cultures of bacteria, that is, cultures in which the number of cells remains relatively constant over an extended period of time, have classically been regarded as cultures in which cell division, and hence growth, is still taking place at a somewhat reduced rate, but in which the death rate of cells approximately equals their rate of multiplication. Such cultures have been considered to be in a state of so-called dynamic equilibrium. Under these circumstances the turnover of insoluble cell material and the incorporation of active carbon from fed compounds should continue. The fact that in the present experiments the rate of turnover was reduced in non-growing cultures by a factor of about three thousand for two such different compounds as glucose and glutamine indicates that the rate of turnover of insoluble cell material, and hence the rate of multiplication and death of cells, was almost negligible. In contrast to the prevailing idea, these non-growing but actively respiring cultures were not in a state of active dynamic equilibrium, but were almost static.

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EFFECT OF FERROCYANIDE ON GROWTH AND ACID PRODUCTION OF *ASPERGILLUS NIGER*[1]

By S. M. Martin

Abstract

Growth of *Aspergillus niger*, in a simple synthetic medium, is inhibited by ferrocyanide ion in concentrations below 1 p.p.m. This inhibition can be reversed by increasing the iron and zinc concentration of the medium. Acid production, however, is inhibited only 50% by 200 p.p.m. ferrocyanide and this inhibition is not reversible by trace metals. Neither the inhibition of growth nor that of acid production is the result of release of cyanide from ferrocyanide. It is suggested that ferrocyanide has at least two functions in the treatment of substrates for use in the production of citric acid. The first is to remove deleterious trace metals from the medium whereas the second is to act as a direct inhibitor of growth of the mold.

Potassium ferrocyanide has been used extensively in the treatment of molasses for the production of citric acid by mold fermentation (2, 4, 5, 6, 9, 11). Its effect has generally been attributed to the removal of deleterious trace metals (1) but there have also been indications that it may exert a direct toxic action on the mold (4, 10). No attempt has been made to study the effect of ferrocyanide under controlled conditions and to determine its mode of action.

Ferrocyanide is quite reactive and several type reactions (1) may be of biological significance:

(I) Reaction with cations ($H_4Fe(CN)_6 + M^+ \longrightarrow$) to yield such salts as $Fe_4[Fe(CN)_6]_3$, $Fe_2Fe(CN)_6$, $Zn_2Fe(CN)_6$, many of which are highly insoluble.

(II) Decomposition in acid solution to yield hydrocyanic acid ($3H_4Fe(CN)_6 \longrightarrow 12HCN + Fe_2Fe(CN)_6$).

(III) Oxidation by atmospheric oxygen in acid solution to yield hydrocyanic acid ($7H_4Fe(CN)_6 + O_2 \longrightarrow 24HCN + Fe_4[Fe(CN)_6]_3 + 2H_2O$).

(IV) Reaction with aldehydes and ketones to yield insoluble addition products.

(V) Reaction (with proteins) in acid solution causing protein precipitation.

Thus in addition to its removal of trace metals, ferrocyanide might be toxic through generation of cyanide (reactions II and III) or it might, in itself, be a toxic agent reacting with an enzyme protein, cofactor, or essential intermediate.

The present communication describes a series of experiments designed to furnish information on the mode of action of ferrocyanide.

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Methods

Aspergillus niger, N.R.C. A1-233, was used throughout this study. Spores from cultures on molasses agar (11), suspended in distilled water, were used to inoculate 500 ml. Erlenmeyer flasks containing 50 ml. of medium. Because of the multitude of side reactions possible in a complex natural medium, a simple, restrictive, synthetic medium was used. This basal medium contained: 140 gm. sucrose, 1.0 gm. potassium acid phosphate, 1.0 gm. ammonium nitrate, 0.25 gm. magnesium sulphate heptahydrate, 6.9 mgm. ferric ammonium sulphate duodecahydrate, 1.1 mgm. zinc sulphate duodecahydrate, hydrochloric acid to pH 3.0, water to 1 liter. The medium, including trace metals (0.018 μM. total Fe^{+++} and Zn^{++} per ml.), was sterilized at 121° for five minutes. Ferrocyanide and extra trace metals were added as indicated in the text, the ratio of iron to zinc being kept constant. The only precaution taken to exclude contaminating trace metals was in the use of reagent grade chemicals. The quiescent cultures were incubated at 28°.

To determine the amount of total acid produced, aliquots of the culture medium were removed at intervals and titrated with 0.1 *N* NaOH (phenolphthalein). Results are expressed as ml. of 0.1 *N* NaOH per ml. of culture filtrate.

Weight of the mold felt was determined after it was washed with hot water and dried under vacuum at 60°.

Free ferrocyanide (i.e. that portion of the original ferrocyanide free to react with ferric iron) was determined in the uninoculated medium by a colorimetric method developed for the purpose. The samples were filtered through a medium porosity sintered glass filter to remove precipitated ferrocyanide complex and an aliquot (5 ml.) of the filtrate, or dilution thereof, added to 5 ml. of 20% citric acid in a test tube. The volume was adjusted to 11 ml., the tube contents mixed, 1 ml. of ferric chloride solution (10% $FeCl_3 . 6H_2O$ and 0.1% gum ghatti in 0.05 *N* HCl) added, and the contents mixed again. The tubes were loosely stoppered and heated for one hour in a water bath at 60°. After heating, the optical density at 675 mμ was determined using a Coleman Junior spectrophotometer. Ferrocyanide standards (30, 60, 90, 120 γ ferrocyanide ion), a sample blank without ferric chloride, and a tube for the determination of recovery of added ferrocyanide were included with each set of samples tested. The ferrocyanide concentration in samples containing very low levels was determined by difference, after adding a known amount of ferrocyanide. The optimum range of the method was about 50 to 100 γ ferrocyanide ion. Recovery of added ferrocyanide ranged from 95 to 105% and duplicate determinations checked within less than 5%. For ease of comparison ferrocyanide and trace metal concentrations have been presented on a molar basis, i.e. μM./ml. of medium.

Experimental and Results

It is important to realize that inhibition or stimulation of acid production may be brought about through an effect on (*a*) growth of the organism, i.e.

on reactions which may or may not bear relationship to acid accumulation, or (*b*) that sequence of reactions leading directly to citrate accumulation. Citric acid production by *A. niger* is limited or absent during growth of the organism (3). It is therefore possible to separate factors affecting acid production from those affecting growth. In the following sections the effect of ferrocyanide on acid production, on growth and acid production, and on growth alone will be considered.

Effect on Acid Production

To test the effect of ferrocyanide on acid production, cultures in the basal medium were incubated until the dry weight of the mold had essentially ceased to increase and a moderate acidity had developed (72 hr.). At that stage varying amounts of ferrocyanide and trace metals were added. Acid production was appreciably inhibited only at high levels of ferrocyanide and increased trace metals were without effect (Table I (*a*)). Ferrocyanide in high concentration (1.0 μM./ml.), added at 72 hr. to cultures in the basal medium, resulted in a general lowering of the rate of acid production (Fig. 1). This effect did not appear to be the result of the liberation of cyanide since, in comparable experiments using cyanide as the inhibitor (Fig. 2), acid production was completely inhibited for varying lengths of time depending upon the cyanide concentration. Following the period of inhibition, acid was produced at a rate equal to, or greater than, in the control. At the end of the incubation period (10 days) all flasks contained approximately equal amounts of acid.

Thus the effect observed with cyanide was quite different from that with ferrocyanide, suggesting that the two were not related. Cyanide, however, is not precluded from contributing to the over-all ferrocyanide effect.

Effect on Growth and Acid Production

When ferrocyanide, at levels as low as 0.05 μM./ml., was added to the basal medium at the time of inoculation, acid production was completely inhibited. However, increasing the trace metals level reversed this inhibition and there was a direct relation between ferrocyanide and trace metals (Table I (*b*)). The accumulation of acid, in media with an intermediate trace metals level (0.09 μM./ml.) and varying amounts of ferrocyanide, suggests that ferrocyanide impedes growth but not acid production (Fig. 3). The lag before commencement of acid production was increased with increasing ferrocyanide, whereas the rate of production was roughly equivalent to control.

Commencement of acid production was delayed when cyanide replaced ferrocyanide (Fig. 4) indicating that cyanide also inhibited growth. Under these conditions, however, the rate of acid production was increased over that of control so that towards the end of the fermentation period cyanide appeared to be without effect or even slightly stimulatory. Also, in contrast to ferrocyanide which inhibited at 0.05 μM./ml., more than 1.0 μM. cyanide/ml. was required to inhibit growth and acid production. Ferrocyanide, therefore, has a function other than as a source of cyanide.

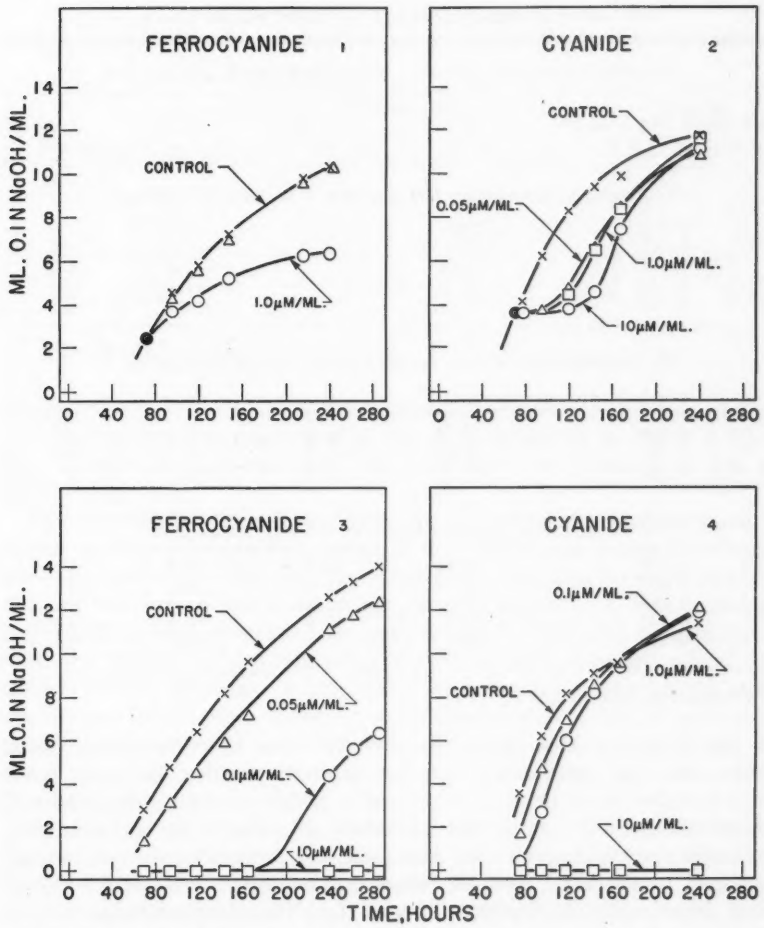

FIG. 1. Effect on acid production of ferrocyanide added 72 hr. after inoculation. (Trace metals, 0.018 μM./ml.)

FIG. 2. Effect on acid production of cyanide added 72 hr. after inoculation. (Trace metals, 0.018 μM./ml.)

FIG. 3. Effect on acid production of ferrocyanide added at time of inoculation. (Trace metals, 0.09 μM./ml.)

FIG. 4. Effect on acid production of cyanide added at time of inoculation. (Trace metals, 0.018 μM./ml.)

TABLE I

EFFECT ON ACID PRODUCTION OF FERROCYANIDE AND TRACE METALS*

Total acid expressed in ml. 0.1 *N* NaOH/ml. culture filtrate

	Ferrocyanide level, μM./ml.			
Trace metals level, μM./ml.	0	0.05	0.10	1.00
(*a*) Ferrocyanide and trace metals added 72 hr. after inoculation				
0.018	10.0		10.0	6.6
0.045	10.8		10.1	6.8
0.090	10.0		10.4	6.8
0.135	10.3		11.0	6.7
0.180	10.8		10.4	6.3
(*b*) Ferrocyanide and trace metals added at time of inoculation				
0.018	10.7	0	0	0
0.045	12.2	7.7	0	0
0.090	12.6	10.8	4.4	0
0.135	11.0	13.0	9.1	0
0.180	11.0	12.8	11.8	0
(*c*) Ferrocyanide and trace metals heated with the medium				
0.018	10.0	12.4	9.0	0
0.045	11.3	13.0	13.1	0
0.090	12.1	13.0	13.0	0
0.135	11.9	12.9	13.0	0
0.180	11.3	12.6	12.3	0

* *Fermentation time, 10 days.*

In the preceding experiments ferrocyanide was added to cooled basal medium following sterilization. In fermentation practice, however, ferrocyanide is added to hot mashes or to mashes which are subsequently heated. To determine the effect on growth and acid production of ferrocyanide when heated with the medium, varying amounts of ferrocyanide and trace metals were added to the basal medium before sterilization. Only at the highest level of ferrocyanide (1.0 μM./ml.) was there complete inhibition of acid production and under most conditions acid production was stimulated (Table I (*c*)). These data suggest that heating greatly reduced the ferrocyanide concentration in the medium.

Effect on Growth

As would be expected from the preceding discussion, the addition of ferrocyanide or cyanide 72 hr. after inoculation affected the dry weight only slightly, growth at this state being virtually complete. However, growth of the mold was very sensitive to ferrocyanide but relatively insensitive to cyanide (Table II). Increasing the trace metals reversed the inhibition of

TABLE II

EFFECT ON GROWTH OF TIME OF ADDITION OF FERROCYANIDE AND CYANIDE*

Dry weight of mold expressed in gm./culture

	Inhibitor and time of addition			
	Ferrocyanide		Cyanide	
Concentration, μM./ml.	0 hr.	72 hr.	0 hr.	72 hr.
0	0.61	0.60	0.58	0.60
0.05	0.01	0.57	—	—
0.10	0.01	0.58	0.62	0.66
0.50	0.01	0.54	0.60	0.63
1.00	0.01	0.52	0.61	0.59
10.00	—	—	0.01	0.59

* *Growth period, 10 days.*

ferrocyanide and there was a direct relation between ferrocyanide and trace metals (Fig. 5). A comparison of the data presented in Table I (*b*) and Fig. 5 indicates that maximum yields of acid are obtained at less than maximum growth.

To determine the lower limit of toxicity of ferrocyanide on growth, varying amounts of ferrocyanide were added at the time of inoculation to cultures in the basal medium (0.018 μM. trace metals/ml.) and after six days' incubation the dry weights of the mold felts were determined. A concentration of 0.005 μM. ferrocyanide/ml. (1 p.p.m.) caused a 70%–90% reduction in the final dry weight while 0.001 μM./ml. was without apparent effect. At a level of 0.0001 μM./ml. (0.02 p.p.m.) ferrocyanide appeared to be slightly stimulatory for growth. However, visual observation of the cultures over the growth period revealed an initial toxic effect even at the lowest level. This effect was very pronounced at 0.001 μM./ml. during the first two days of incubation.

To determine whether ferrocyanide inhibited spore germination or vegetative growth, ferrocyanide (0.01 μM./ml.) was added to cultures in the basal medium at intervals during the first 48 hr. of incubation. Dry weight of the mold after six days (Fig. 6) clearly indicated that ferrocyanide did not act simply as an inhibitor of spore germination.

It has been observed (4, 10) that, in molasses treated with ferrocyanide, *A. niger* failed to grow if the pH of the medium was too low. The effect of pH on ferrocyanide toxicity was tested by adjusting the reaction of the basal medium toward neutrality with 20% KOH and adding ferrocyanide at the time of inoculation. The increased dry weight of the mold at higher pH values (Fig. 7) indicated that ferrocyanide toxicity was pH sensitive. Under the conditions employed, however, it is only moderately so, growth at pH 7.0 still being strongly inhibited. The reaction of the medium did not affect the weight of the control cultures.

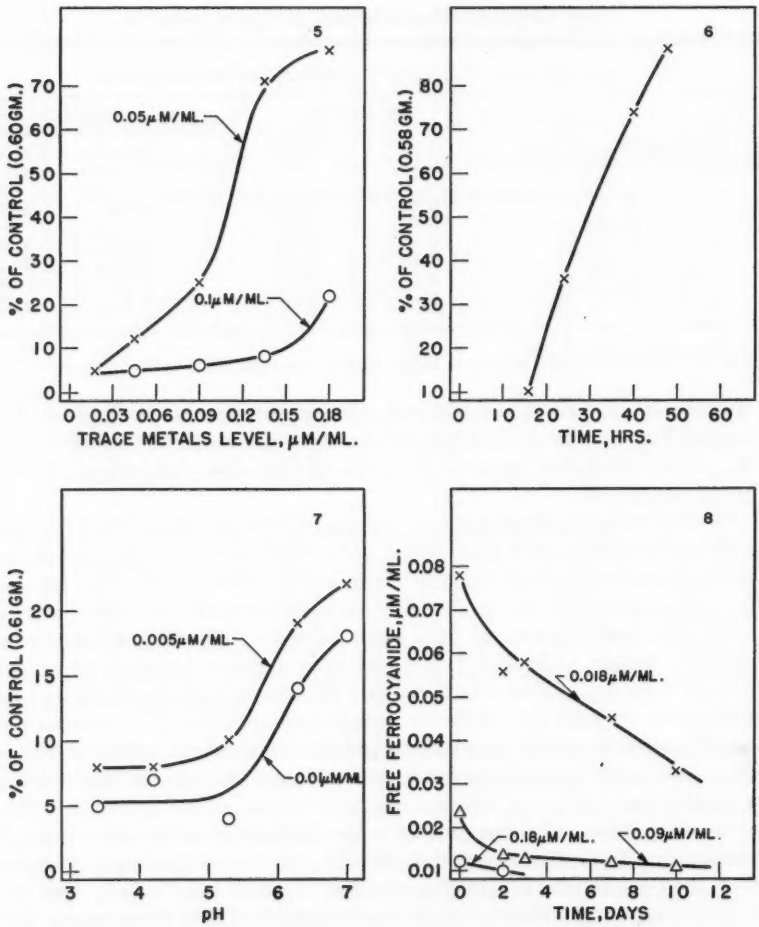

FIG. 5. Reversal of ferrocyanide toxicity for growth by trace metals.

FIG. 6. Effect on growth of time of addition of ferrocyanide. (Ferrocyanide, 0.01 μM./ml.; trace metals, 0.018 μM./ml.)

FIG. 7. Effect of pH on inhibition of growth by ferrocyanide. (Trace metals, 0.018 μM./ml.)

FIG. 8. Effect of time and trace metals on free ferrocyanide. (Ferrocyanide, 0.1 μM./ml.)

Free Ferrocyanide

When ferrocyanide was added to the medium a blue color developed, which intensified on standing, indicating that ferrocyanide was being removed from solution. To study the disappearance of the inhibitor from the medium, flasks of sterile medium containing different levels of ferrocyanide and trace metals were assayed for free ferrocyanide after varying lengths of time. The data (Fig. 8) indicate that after the initial rapid drop in ferrocyanide concentration (occurring in less than one hour) there was a gradual decline continuing throughout the experiment. In the presence of increased trace metals, the ferrocyanide concentration was soon below the limits of measurement. When ferrocyanide was heated in the medium, the initial decrease was still greater. Thus when 1.0 μM./ml. was heated in the basal medium 85% of the ferrocyanide was lost. The concentration was below the limits of measurement when 0.1 μM. was similarly tested.

These data help to explain why, after an initial inhibition of growth, the organism is able to grow, provided the concentration of added ferrocyanide is low enough and the trace metals level is high enough. They also explain the differences observed in growth and acid production between heated and unheated ferrocyanide.

Discussion

The growth process in *A. niger* is extremely sensitive to ferrocyanide whereas the sequence of reactions leading to acid production is relatively insensitive. The inhibition of growth does not appear to be the result of cyanide arising from ferrocyanide since it requires a molar concentration of cyanide approximately 100 times that of ferrocyanide to inhibit growth. To determine, however, if growth inhibition is a direct effect of ferrocyanide or a secondary effect on trace metals is more difficult. In general, however, the data suggest that there is an important direct effect. For example, ferrocyanide at extremely low levels, or at higher levels in the presence of increased trace metals, causes a stimulation of growth and resultant acid production. This effect does not appear to be the result of reduction of trace metals level since the data indicate that the basal medium was somewhat deficient in this respect, optimum growth and acid production occurring at the intermediate metals level (0.09 μM./ml.) in the absence of ferrocyanide. This general effect was very pronounced in experiments where ferrocyanide and trace metals were heated with the medium.

The specific effect of ferrocyanide may be the result of inhibition of isocitric dehydrogenase since it has been shown (7) that this enzyme, from *A. niger*, is ferrocyanide sensitive but not cyanide sensitive. It has not been demonstrated, however, that this is the only site involved and it might be reasoned that, because of the extreme toxicity of the ion, a still more critical reaction is involved.

In considering the effect of ferrocyanide on a biological system it is of the utmost importance to recognize the effect of composition and treatment of the medium. As has already been shown, trace metals level, temperature,

and time are factors in determining the amount of ferrocyanide remaining in the medium. Thus in the synthetic medium used above, effects on growth were noted at levels below 1 p.p.m., whereas in molasses mashes (unpublished results) a minimum of about 100 p.p.m. free ferrocyanide is required for good fermentations and the mold will tolerate 500 p.p.m. or more.

On the basis of the information at hand, it can be postulated that ferrocyanide has at least two functions when used in the treatment of molasses, both instrumental in controlling growth of the organism. The first is the removal of trace metals which favor growth but not acid production, which is in accord with the observations (8, 12) that good fermentations can be achieved through the use of metal deficient media. The second, and perhaps more important, is the direct effect of ferrocyanide on the mold, which is supported by the observation that in molasses a moderately high residual ferrocyanide level is required for satisfactory fermentations (unpublished results). At a pH near neutrality ferrocyanide toxicity is reduced and growth occurs. However, the mold soon begins to produce acid and, with the lowering of the pH, toxicity increases until further growth is stopped. A situation then exists where growth, which is incompatible with acid production, is inhibited by an agent which has no effect on acid production.

It seems reasonable to assume that ferrocyanide may be used to control the growth of *A. niger* when substrates other than molasses are used for the production of citric acid. However, a careful study of the effect of ferrocyanide relative to the particular substrate would be necessary.

Acknowledgment

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UTILIZATION OF SODIUM CASEINATE BY SOME HOMOFERMENTATIVE LACTOBACILLI[1]

By I. J. McDonald

Abstract

Species of homofermentative lactobacilli commonly associated with dairy products grew in chemically defined basal medium containing sodium caseinate while species from other sources did not. The utilization of sodium caseinate may be a useful differential characteristic in the classification of lactobacilli.

Introduction

In descriptions of species of the genus *Lactobacillus* by Breed *et al.* (1), *L. casei* is reported to utilize casein, *L. bulgaricus* and *L. caucasicus* are reported to be without action on casein in milk, while the action of other species of homofermentative lactobacilli on casein is not reported. During an investigation on the utilization of sodium caseinate by strains of *L. casei* (6), it became of interest to determine the ability of several other species of homofermentative lactobacilli to grow in a chemically defined basal medium with unhydrolyzed sodium caseinate as the source of amino acids.

Materials and Methods

Cultures of lactobacilli (Table I) were maintained by weekly transfer in milk or milk plus glucose.

Utilization of unhydrolyzed sodium caseinate by lactobacilli was determined in the basal medium described previously (6) with the following additions per 5.0 ml. of double strength medium (Medium I): glucose 200 mgm., xanthine 0.2 mgm., orotic acid 0.25 mgm., vitamin B-12 0.0075 μgm., leucovorin (calcium salt) 2.5 μgm., pantethine 2.5 μgm., and thymidine 2.5 μgm. Immediately before sterilization of the basal medium at 121° C. for 15 min., cysteine hydrochloride was added so that the concentration in the complete medium was 0.0158 mgm./ml. Hydrolyzed sodium caseinate was prepared by adding 10 ml. of a 0.1% pancreatin solution to 100 ml. of a 2% solution of sodium caseinate (6). As a control, pancreatin solution, inactivated by boiling for 10 min., was added to unhydrolyzed sodium caseinate solution. These solutions were Seitz filtered, incubated 24 hr. at 30° C., boiled for 10 min., and added to sterile basal medium so that the final concentration was the equivalent of 2.0 mgm. of sodium caseinate/ml. Cells used as inocula were from second serial subcultures in Medium I supplemented with casitone (Difco) 0.1%, casamino acids (Difco) 0.1%, and yeast extract (Difco) 0.1%. Cells from 24-hr. cultures were centrifuged, washed three times, and diluted to 10 times their original volume in 0.9% saline. An inoculum of 0.1 ml.

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TABLE I

CULTURES OF *Lactobacilli*

Organism	Number	Usual habitat*	Obtained from
L. casei	BC-1	Dairy products	Univ. of Wisconsin
" "	7469	" "	A.T.C.C.
" "	B-49, B-50	" "	Univ. of British Columbia
L. bulgaricus	12	" "	Dr. M. Rogosa
" "	Ga, Sarles	" "	Univ. of Wisconsin
L. lactis	X-37	" "	" " "
L. helveticus	80X	" "	Dr. M. Rogosa
" "	H-77	" "	Dr. E. E. Snell
L. plantarum	4943	Dairy and plant products	A.T.C.C.
" " †	B-45, B-46, and B-48	" " "	Univ. of British Columbia
L. arabinosus‡	17/5	" " "	Univ. of Wisconsin
L. pentosus‡	8041	" " "	A.T.C.C.
L. acidophilus	206X	Feces of milk fed infants	Dr. M. Rogosa
" "	4357, 9857	" " " "	A.T.C.C.
" "	630, 746	" " " "	Science Service, Ottawa
L. delbrueckii	730	Vegetable and grain mashes	Dr. M. Rogosa
" "	LD-3	" " " "	Univ. of Wisconsin
L. leichmannii	313	Plant and dairy products	Dr. M. Rogosa
" "	4797	" " " "	A.T.C.C.
" "	643	" " " "	Science Service, Ottawa

* *Bergey's Manual of Determinative Bacteriology* (*1*).
† *Isolated from cheese* (*3, 9*).
‡ *Isolated from vegetable source* (*4*).

was used per 10 ml. of medium. After incubation the lactic acid produced by cultures was titrated with 0.1 *N* sodium hydroxide to the phenolphthalein end point.

Cultures of lactobacilli were classified using the scheme of Rogosa *et al.* (8). Organisms were grown in the following medium (Medium II): glucose 2%, tryptone (Difco) 0.5%, tryptose (Difco) 0.5%, yeast extract (Difco) 0.5%, dipotassium phosphate 0.5%, sodium acetate 1.0%, citric acid 0.1%, ammonium hydroxide 0.09%, magnesium sulphate heptahydrate 0.02%, manganous sulphate monohydrate 0.04%, ferrous sulphate heptahydrate 0.001%, and polyoxyethylene sorbitan monooleate (Tween 80) 0.1%. The medium was adjusted to pH 6.0 before sterilization. Inocula were from 24-hr. cultures in this medium. Cells were centrifuged, washed, and resuspended to their original volume in 0.9% saline and 0.1 ml. was used per 10 ml. of medium. Growth at 16° and 45° C. was determined after incubation for 10 days. Fermentation of Seitz filtered carbohydrates was determined using bromocresol purple indicator in Medium II without glucose. The lactic acid produced in Medium II was isolated as the zinc salt (2) and optical rotations were determined on 2% solutions. The acidity produced by lactobacilli in reconstituted skim milk (Difco) was determined after incubation for 10 days at their optimum growth temperature. Aliquots were titrated with 0.1 *N* sodium hydroxide using phenolphthalein indicator. The morphology of colonies was determined using tryptone glucose extract agar (Difco) plus 0.5% yeast extract (Difco).

Results

It was shown previously (6) that, in the presence of cysteine, strains of *L. casei* utilized unhydrolyzed sodium caseinate as a source of amino acids. Strains of *L. bulgaricus*, *L. helveticus*, *L. lactis*, and *L. plantarum* usually associated with dairy products also utilized this protein in the presence of cysteine (Table II) but growth was less rapid than with hydrolyzed caseinate. In the latter medium, acid production was essentially complete after 40 hr. incubation (results not shown). After 66 hr. incubation in sodium caseinate medium, most caseinate utilizing strains produced 50 to 90% as much acid as in hydrolyzed caseinate but *L. plantarum* strains B-45, B-46, and B-48 produced only 25% as much. In caseinate medium in the absence of cysteine, most caseinate utilizing lactobacilli grew poorly and thus resembled *L. casei*. However, the growth of *L. plantarum* strain B-48 was almost the same in the presence or absence of cysteine.

Lactobacilli not usually associated with dairy products also were tested for their ability to utilize sodium caseinate. *L. pentosus*, *L. arabinosus*, and some strains of *L. acidophilus*, *L. delbrueckii*, and *L. leichmannii* did not utilize this protein (Table III), the amount of acid produced being 15% or less of the amount produced with hydrolyzed caseinate. However, some strains of the latter three organisms did utilize caseinate almost as readily as the hydrolyzate.

TABLE II

UTILIZATION OF SODIUM CASEINATE BY LACTOBACILLI USUALLY ASSOCIATED WITH DAIRY PRODUCTS*

Titratable acidity (as % lactic acid)† (66-hr. incubation)

Organism		Sodium caseinate	Pancreatin hydrolyzed sodium caseinate
L. casei	BC-1	1.30	1.35‡
" "	7469	0.90	1.40
" "	B-49	0.40‡	0.70‡
" "	B-50	0.48	—
L. *plantarum*	4943§	0.97	1.01
" "	B-45	0.22	0.91
" "	B-46	0.27	1.02
" "	B-48	0.26	1.05
L. bulgaricus	Sarles	0.90	1.22
" "	Ga	0.82	0.85
" "	12	0.70	1.31
L. helveticus	80X	1.05	1.47
" "	H-77§	0.99	—
L. lactis	X-37	0.25‡	0.52‡

* *Medium I (text) plus 2.0 mgm. sodium caseinate or the equivalent as hydrolyzed caseinate and 0.0158 mgm. cysteine hydrochloride/ml. of final medium. L. plantarum strains incubated at 30° C., remainder at 35° C.*

† *Average of three to six determinations.*

‡ *Forty-hour incubation.*

§*Reclassified as L. casei (see Table IV).*

TABLE III

UTILIZATION OF SODIUM CASEINATE BY LACTOBACILLI NOT USUALLY ASSOCIATED WITH DAIRY PRODUCTS*

Titratable acidity (as % lactic acid)† (66-hr. incubation)

Organism		Sodium caseinate	Pancreatin hydrolyzed sodium caseinate
L. acidophilus	206X	0.09	0.59‡
" "	4357	0.04	0.43
" "	9857	0.02	1.06
" "	630§	*0.90*	0.81
" "	746§	*0.91*	1.08
L. delbrueckii	730	0.06	1.21
" "	LD-3§	*1.28*	1.48
L. leichmannii	313	0.06	0.69
" "	4797	0.03	0.27
" "	643§	*0.81*	1.08
L. arabinosus	17/5	0.14	1.41
L. pentosus	8041	0.06	0.86

See footnotes to Table II.

TABLE IV

CHARACTERISTICS OF LACTOBACILLI RECLASSIFIED AS *L. casei**

Organism		Colony morphology	% lactic acid (milk)	Rotation of lactic acid	Growth at:	
					45° C.	16° C.
L. casei†		S	0.09–1.7	D	+ or 0	+
L. plantarum†		S	0.3–0.8	I	0	+
L. helveticus	H-77	S	1.08	D	+	+
L. acidophilus	630	S	0.7	D	+	+
L. acidophilus	746	S	0.7	D	+	+
L. leichmannii	643	S	0.7	D	0	+
L. delbrueckii	LD-3	S	0.05	D	+	+
L. plantarum	4943	S	0.17	D	+	+

* *S = Smooth colony, D = dextrorotatory lactic acid, I = inactive (DL) lactic acid, + = growth, 0 = no growth.*

† *Characteristics of L. casei and L. plantarum as given by Rogosa et al. (8).*

Remainder of species named as received. Their carbohydrate fermentations were the same as those given for L. casei (8).

Since cultures received as the same species did not always react towards casein in the same way, all cultures were classified according to the scheme of Rogosa *et al.* (8). On the basis of colony morphology, acidity produced in milk, rotation of lactic acid, and fermentation of carbohydrates, *L. acidophilus* 630 and 746, *L. delbrueckii* LD-3, *L. leichmannii* 643, *L. helveticus* H-77, and *L. plantarum* 4943 apparently were strains of *L. casei* (Table IV). The ability of these cultures to utilize casein (Tables II and III) also indicated they were strains of *L. casei*. It should be noted that *L. delbrueckii* LD-3 failed to clot milk or ferment lactose and *L. plantarum* 4943 clotted milk and fermented lactose slowly. These organisms, therefore, appeared to be similar to *L. casei* var. *alactosus* Rogosa *et al.* (8). *L. plantarum* B-48 produced inactive lactic acid, a characteristic of this species (8), but failed to ferment melibiose, a characteristic of *L. casei* (5, 7). This culture thus appeared to be intermediate between *L. plantarum* and *L. casei* and may be related to similar strains discussed by Sherwood (10).

Seven of 26 strains were evidently misnamed. At least one strain was incorrectly named when received but it is not known if the others were incorrectly identified originally or became contaminated at some time during their maintenance.

Discussion

The ability of homofermentative lactobacilli to utilize unhydrolyzed sodium caseinate as a source of amino acids seems to be confined to organisms usually associated with dairy products, and this may have some significance. Since *L. casei* is considered important in cheese ripening because it utilizes casein (1), strains of *L. bulgaricus*, *L. helveticus*, *L. lactis*, and *L. plantarum* may also be important in this process. It is interesting that sodium caseinate was utilized by *L. plantarum* strains (B-45, B-46, and B-48) isolated from cheese (3, 9) but was not utilized by *L. arabinosus* and *L. pentosus* isolated from vegetable sources (4). The latter two organisms have been classified as *L. plantarum* by Breed *et al.* (1) but the above observations appear to support the suggestion of Rogosa *et al.* (8) that *L. arabinosus* constitutes a distinct species.

Although the results presented above were obtained with a limited number of organisms, it would appear that the utilization of sodium caseinate may have some value as a characteristic for classification of lactobacilli. Thus, as pointed out above, strains of *L. plantarum* may be divided into two groups, those that utilize sodium caseinate (*L. plantarum*) and those that do not (*L. arabinosus*). The inability of *L. acidophilus* to utilize sodium caseinate together with characteristics published recently (8, 11) may serve to differentiate it more readily from *L. casei* and *L. bulgaricus*. Also strains of *L. casei* var. *alactosus* (8) may be distinguished more readily from the non-lactose fermenting *L. delbrueckii* and *L. leichmannii* on the basis of the ability of the first and the inability of the latter two organisms to utilize sodium caseinate.

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THE UTILIZATION OF PEPTIDES AND L- AND D-AMINO ACIDS BY EFFECTIVE AND INEFFECTIVE STRAINS OF *RHIZOBIUM MELILOTI*[1]

By D. C. Jordan and C. L. San Clemente

Abstract

Washed cells of three strains of *Rhizobium meliloti*, effective and ineffective in the fixation of atmospheric nitrogen, were found to utilize certain di- and tripeptides and the L-, D-, and DL-forms of several amino acids as sole sources of nitrogen. The maximum growth attained on any one amino acid isomer was a function of pH and temperature. Inhibitory effects found in certain racemic mixtures were explained by mutual stereochemical interference of the enzymes attacking the L- and D-isomers. Histidine was notable in that it yielded the greatest amount of growth at all pH values (6.0, 7.0, 7.5) with all three organisms, including the ineffective strain which did not grow at pH 6.0 or 7.5 on any other acid. All strains were relatively acid sensitive, variable degrees of growth inhibition occurring at pH 6.0. Partial hydrolysis of the peptides occurred, but stimulatory effects were observed in many instances and explained on the basis of transpeptidation. Inhibition in media containing peptides of glycine plus D-leucine was traced to an antagonistic effect between the two constituent amino acids after liberation. Although the ineffective and effective strains of rhizobia were found to differ in several ways, the most interesting feature was the stimulation of the growth of the ineffective organism by D-leucylglycine.

Introduction

It has been previously shown (2) that washed cells of alfalfa – sweet clover rhizobia are capable of utilizing any one of a large number of L- and DL-amino acids as a sole source of nitrogen. Unfortunately, the growth on racemic mixtures of amino acids may be complicated by the differential biological effects of the D- and L-isomers present. This problem can be partially resolved by comparing the growth-promoting abilities of L-, D-, and DL-amino acids. Such an investigation, coupled with observations on the effects of pH and temperature on amino acid utilization, was therefore initiated. It was hoped that such experimentation would also yield data that would be useful for (*a*) the eventual development of a synthetic minimal medium which could be employed in the study of auxotrophic mutants of these organisms and for (*b*) extending the search for fundamental biochemical differences among strains differing in nitrogen-fixing ability. This search, which so far has yielded only negative results, is of considerable importance. Knowledge concerning the basic reasons for effectiveness, ineffectiveness, and parasitism within the genus *Rhizobium* would aid in the formulation of a rapid test for strain efficiency, in the elucidation of the symbiotic relationship, and, perhaps, in clarification of the mechanism of fixation itself.

Since there are now a number of reports (5, 7) dealing with the stimulation of bacterial growth by peptides it was felt that the beneficial results of such

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substances might also be manifested in rhizobia. Therefore, several experiments were devised to ascertain the presence and extent of such activity, particularly in regard to peptides containing either L- or D-leucine.

Methods

Rhizobium meliloti strains R_{21} and $R_{20\text{-}27A}$ (effective in nitrogen fixation) and R_{20} (ineffective to parasitic)* were subjected to single-cell isolation and the resulting clones statistically tested for nitrogen-fixing ability on Ontario Variegated alfalfa, using the sterile glass assemblies of Leonard (6) and the techniques previously described by Jordan and Garrard (4). Strain $R_{20\text{-}27A}$ was a colonial mutant of R_{20} obtained by X-ray irradiation (1).

The synthetic basal medium employed was that of Wilson and Wilson (10) modified by the exclusion of ammonium chloride. It was adjusted to a pH level of either 6.0, 7.0, or 7.5 (a pH higher than this caused turbidity) and sterilized by Seitz filtration. The first 20 ml. of medium passing through the filter were discarded and 9.5 ml. portions of the remainder were aseptically added to sterile chemically-clean cuvettes. Stock solutions of amino acids and peptides† were prepared with double glass-distilled water, adjusted to pH 7.0 where necessary, sterilized, and their purity established by descending paper-partition chromatography in water-saturated phenol (12). These solutions were added to their respective cuvettes in 0.5 ml. quantities to yield media containing the equivalent of 0.0001 moles of nitrogenous compound per liter. The peptide solutions were added to pH 7.0 basal medium only.

Ten-milliliter amounts of a modified mineral salts – yeast extract medium (11) were inoculated with the test organism and incubated at 30° C. for 24 hr. The cells were then centrifuged, washed three times in 0.85% saline, diluted to nephelos 55.0 (0.1 mgm. bacterial nitrogen per ml.), and 0.2 ml. amounts of the resulting suspension used for cuvette inoculation. Thus the total initial cuvette volume was 10.2 ml. and was experimentally determined to take into consideration the extent of evaporation which occurred during the incubation period.

In each experimental series one strain of *Rhizobium* was tested for its ability to utilize amino acids and peptides as sole nitrogen sources. Incubation was carried out at 30° C. and inoculated and uninoculated control cuvettes, containing no added combined nitrogen, were employed. The uninoculated control served as a blank for the readings which were taken daily in a Coleman photonephelometer. This particular instrument gives readings in terms of nephelos units, which refer to the Tyndall light reflected from the bacterial cells. After the better pH for growth on any particular L- or D-amino acid had been established, this same acid was retested at this pH, but at a temperature of 27° C.

* *This strain behaves as a parasite with some hosts (4), but is only ineffective with others.*
† *Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.*

It was statistically calculated that deviations in nephelos from the true value were not related to the nephelos magnitude. Since, however, significance at the 5% point requires a difference exceeding ± 1.96 standard deviations, an arbitrary difference of four such deviations between any two readings was required for significance. This amounted to about eleven nephelos units (four times the mean standard deviation of $2.69 = 10.76$).

A cell-free extract of the effective strain R_{21}, containing 0.41 mgm. nitrogen per ml., was prepared as previously described (3) and 0.5 ml. amounts added to equal volumes of solutions containing 5 mgm. of L- and D-leucylglycine or leucylglycylglycine. After one hour at 30° C., 0.04 ml. samples of the mixtures were analyzed by paper-partition chromatography in water-saturated phenol. Controls included cell-free extract alone, known amino acids and peptides, test solutions sampled at zero time, and test solutions containing known amino acids. The developed chromatograms were sprayed with 0.1% alcoholic ninhydrin and examined for evidence of peptide hydrolysis.

Results

Tables I and II indicate the effects of pH and temperature on amino acid utilization. All three bacterial strains were capable of using the L-, D-, or DL-amino acids as nitrogen sources and, as might be expected, the ability of these compounds to produce growth varied with pH and temperature. A pH of 7.5 was better for the growth of both effective strains, while the ineffective strain was relatively fastidious in that it grew only at 7.0 with all the amino acids except histidine, which was capable of being utilized at each of the pH's tested. The mutant, $R_{20\text{-}27A}$, was the most acid tolerant strain, growth occurring at pH 6.0 with every compound. Histidine was the most remarkable amino acid in that it produced the greatest growth of all three organisms at all pH values.

In regard to temperature, the growth at 30° C. was better than that at 27° C. (when compared at optimum pH) with several exceptions, such as that observed with the effective mutant, $R_{20\text{-}27A}$, on L-glutamic acid. In addition, no significant differences could be found between the effects of the two different temperatures on the utilization of glycine and L-methionine by $R_{20\text{-}27A}$ and glycine and L-glutamic acid by the ineffective organism R_{20}.

Growth in certain racemic mixtures provided evidence for the occurrence of both inhibition and stimulation when the observed and calculated growth maxima were compared (Table III).

As indicated in Table IV all the peptides tested served as nitrogen sources and, in most instances, inhibitory or stimulatory activities were located when the observed and calculated nephelos values were compared. Except for an over-all reduction in the magnitude of growth (and in the growth on L-leucylglycylglycine as compared with L-leucylglycine), the response pattern of the mutant was similar to that of the other effective organism. The pattern for the ineffective bacterium was noticeably different in that superior growth occurred on tri-glycine and D-leucylglycine as compared with L-leucylglycine.

TABLE I

INFLUENCE OF pH ON AMINO ACID UTILIZATION AT 30° C.

Amino acid	Maximum growth in nephelos units								
	R_{21}* (effective)			R_{20}* (ineffective)			$R_{20\text{-}27A}$* (effective mutant)		
	pH 6.0	pH 7.0	pH 7.5	pH 6.0	pH 7.0	pH 7.5	pH 6.0	pH 7.0	pH 7.5
Glycine	0.0	23.0	141.0	0.0	34.0	0.0	17.0	38.2	38.0
L-Leucine	0.0	170.2	181.0	0.0	74.5	0.0	17.0	80.9	115.0
D-Leucine	0.0	195.0	214.5	0.0	156.5	0.0	24.0	48.6	136.3
DL-Leucine	0.0	168.7	188.0	0.0	99.0	0.0	58.5	76.6	91.0
L-Valine	11.5	194.0	224.5	0.0	115.0	0.0	37.7	67.0	95.5
D-Valine	0.0	160.0	193.5	0.0	67.5	0.0	11.5	79.9	56.0
DL-Valine	0.0	168.0	184.5	0.0	76.0	0.0	67.5	70.0	35.2
L-Methionine	16.2	156.0	200.0	0.0	145.5	0.0	92.5	110.2	120.5
D-Methionine	12.0	186.0	160.0	0.0	64.0	0.0	44.0	92.1	68.8
DL-Methionine	11.5	202.0	189.2	0.0	34.3	0.0	44.0	59.3	77.5
L-Glutamic acid	9.5	140.0	207.5	0.0	76.5	0.0	106.0	136.5	140.5
D-Glutamic acid	0.0	130.0	148.5	0.0	93.7	0.0	35.5	71.2	65.5
DL-Glutamic acid	0.0	116.8	132.2	0.0	56.0	0.0	39.5	88.9	69.5
L-Histidine	378.5	371.6	390.0	355.0	449.5	469.5	334.5	395.0	467.5
D-Histidine	462.0	438.5	345.0	410.0	380.0	340.2	390.0	382.0	399.2
DL-Histidine	381.5	369.0	312.0	460.5	461.0	385.0	462.5	366.2	357.5

* *Strain of Rhizobium meliloti.*

TABLE II

INFLUENCE OF 27° C. INCUBATION TEMPERATURE ON AMINO ACID UTILIZATION AT "OPTIMUM" pH

Amino acid	Maximum growth in nephelos units		
	R_{21}* (effective)	R_{20}* (ineffective)	$R_{20\text{-}27A}$* (effective mutant)
Glycine	134.0	36.0	49.0
L-Leucine	165.2	40.0	84.0
D-Leucine	146.0	40.0	80.0
L-Valine	100.0	8.0	44.0
D-Valine	125.0	25.0	36.0
L-Methionine	86.0	144.0	71.0
D-Methionine	69.0	54.0	46.0
L-Glutamic acid	191.4	141.0	151.0
D-Glutamic acid	122.0	66.0	33.0
L-Histidine	321.6	226.0	195.0
D-Histidine	337.0	241.0	173.0

* *Strain.*

TABLE III

INHIBITION AND STIMULATION FOUND IN THE RACEMIC MIXTURES OF CERTAIN AMINO ACIDS

Strain	pH	Inhibition	Stimulation
R_{21} (effective)	7.0	Histidine (91.1%)* Glutamic (86.5%)	
	7.5	Histidine (84.9%) Glutamic (74.3%)	
$R_{20\text{-}27A}$ (effective mutant)	6.0	Methionine (64.5%) Glutamic (55.9%)	Leucine (285.4%) Valine (272.5%) Histidine (127.7%)
	7.0	Methionine (58.6%) Glutamic (85.6%)	
	7.5	Methionine (53.3%) Glutamic (67.5%) Leucine (72.4%) Valine (46.5%)	
R_{20} (ineffective)	6.0	—	Histidine (121.5%)
	7.0	—	Histidine (111.2%)

* *Numbers in parentheses refer to the per cent of the calculated growth. This calculated growth is equal to one-half the growth on the L-isomer + one-half the growth on the D-isomer at the same pH, assuming no inhibition or stimulation.*

TABLE IV

STIMULATION AND INHIBITION OF *Rhizobium* GROWTH BY SYNTHETIC PEPTIDES

	Effective strain (R_{21})			Effective strain ($R_{20\text{-}27A}$)			Ineffective strain (R_{20})		
	Obs.	Calc.	Result	Obs.	Calc.	Result	Obs.	Calc.	Result
Glycine	23.0			38.2			34.0		
Glycylglycine	308.0	46.0	S(262.0)	120.8	76.4	S(44.4)	277.5	68.0	S(209.5)
Glycylglycylglycine	302.0	69.0	S(233.0)	107.4	114.6	N.S.D.	426.5	102.0	S(324.5)
L-Leucine	170.2			80.9			74.5		
L-Leucylglycine	466.0	193.2	S(272.8)	363.5	119.1	S(244.4)	379.0	108.5	S(270.5)
L-Leucylglycylglycine	483.0	216.2	S(266.8)	153.8	157.3	N.S.D.	187.2	142.5	S(44.7)
D-Leucine	195.0			48.6			156.5		
D-Leucylglycine	54.0	218.0	I(164.0)	37.2	86.8	I(49.6)	302.0	190.5	S(111.5)
D-Leucylglycylglycine	60.0	241.0	I(181.0)	70.2	125.0	I(54.8)	208.0	224.5	I(15.8)

Obs. = *Observed values* = *The maximum nephelos readings attained in an N-free synthetic medium containing these peptides or amino acids.*
Calc. = *Calculated values* = *Values obtained by assuming complete hydrolysis of the peptide. For example, the calculated value for glycylglycine would be twice the observed value for glycine.*
S = *Stimulation*, I = *Inhibition. The magnitude of such activity is indicated by the nephelos values in parentheses.*
N.S.D. = *No significant difference.*

This particular strain also made better use of those peptides which contained D-amino acids. The R_f values at 27° C. for glycine, leucine, di-glycine, tri-glycine, L- (and D-) leucylglycine, and L- (and D-) leucylglycylglycine were 0.41, 0.82, 0.42, 0.48, 0.76, and 0.79 respectively. The cell-free bacterial extract at least partially hydrolyzed the peptides against which it was tested, glycine being found as the free acid.

Discussion

In consideration of the report of Rydon (8), who lists a number of microorganisms that are able to use D-amino acids, the bacterial strains employed in the present investigation cannot be regarded as very unusual with respect to their activity towards these "unnatural" isomers. This same worker mentions, however, that of thirty-one bacterial species examined only five, including *Rhizobium leguminosarum*, have so far not been found to use at least one D-amino acid. More work is required to determine whether or not the ability to utilize D-amino acids represents a major difference among the various species of rhizobia.

The effects of pH and temperature on growth in the presence of any individual amino acid may be a reflection of the action of these factors on the enzymes concerned in the metabolism of this compound. (In addition, changes in pH can affect the degree of ionization of constituents of the cellular boundary-layer and hence can result in permeability changes.) Since it would be expected that a number of different routes of amino acid metabolism would exist in intact *Rhizobium* cells it would be hazardous to make any specific statements as to the effects of such factors as pH and temperature on the utilization of any one amino acid. Nevertheless, the results do stress the importance of indicating the medium composition when reporting the "optimum" temperature or pH for any organism.

The outstanding ability of histidine to serve as a nitrogen source may be a result of a difficulty on the part of the organism in synthesizing it from other amino acids. It would then act as a limiting factor in growth unless supplied preformed in the medium. At any rate the mechanism concerned in histidine utilization was more resistant to pH changes than that concerned with any of the other amino acids, particularly in the case of the ineffective strain of *Rhizobium*. This acid shows the greatest promise as a single nitrogen source for the growth of these strains of nodule bacteria.

With compounds as similar as the L- and D-isomers of amino acids it is not surprising that inhibition was observed in many racemic mixtures. If stereospecific enzymes attack the two different isomers of an amino acid, then these isomers are potential substrate-analogues and could act as mutual inhibitors. The extent of inhibition would vary statistically depending upon the relative proportions of competing amino acid and enzyme molecules. The stimulation found in the racemic mixtures of several amino acids at the lower pH values is inexplicable at present. These findings emphasize the fact that it is a fallacy to use DL-amino acids in microbiological experiments under the assumption that the D-isomer is biologically inert.

Although hydrolysis of the peptides by cell-free bacterial extracts was demonstrated, the data are not consistent with the concept of complete hydrolysis. The di- and tri-peptides of glycine and glycine plus L-leucine, with two exceptions, stimulated the growth of the three *Rhizobium* strains above the values that would be expected if complete hydrolysis were assumed. The two exceptions occurred with the mutant $R_{20\text{-}27A}$ and the peptides triglycine and L-leucylglycine, both of which yielded observed growth values which were not significantly different from values calculated on the basis of complete peptide degradation. If it is assumed that the stimulative peptides can be directly utilized for protein synthesis by rhizobia the amount of observed stimulation is probably representative of a balance between the rate of incorporation of the peptides into cellular proteins and the relative peptidase activity of these microorganisms.

The inhibitory effect noted when the two effective strains were grown in the presence of the D-leucine–glycine peptides may partly be the result of an inability to carry out transpeptidation reactions involving D-amino acids. If, however, incorporation did not occur by reason of the contained D-amino acids it would have been expected that hydrolysis would have proceeded to completion, the amino acid products being removed from the site by various types of secondary reactions. With such hydrolysis the observed growth of the organism should have been equal to the calculated growth with no evidence of inhibition. The fact that inhibition was present made it appear that one of the liberated amino acids had blocked the utilization of the other or else that a mutual inhibition had taken place. Confirmation of such activity was obtained by comparing the growth of washed R_{21} cells in basal medium containing either glycine, D-leucine, or glycine plus D-leucine. A definite reduction in growth was found in the medium containing both amino acids when its maximum nephelos was compared with the calculated. This action may be similar to that observed by Kihara and Snell (5) who showed that high levels of glycine inhibited the utilization of D-alanine by *Lactobacillus casei*, but did not affect L-alanine.

Since *Rhizobium* R_{20} was stimulated by D-leucylglycine one might postulate incorporation of this peptide into the protein of the bacterium, but the occurrence of other unknown mechanisms must first be investigated.

The peptidase activity of these rhizobia appears to be somewhat similar to that of *Leuconostoc mesenteroides* which Smith (9), in his review article, states as having two dipeptidases and at least two tripeptidases. In addition, this same organism was apparently found to exert a rapid action on the D-forms of a variety of peptides, an activity which it is now known to share with certain strains of *Rhizobium*.

It is unknown whether or not the differences observed among the ineffective and effective strains were associated with the condition of ineffectiveness, but such differences may be adequately explained by strain variation as it normally occurs among bacteria. Nevertheless, the preferential stimulation of the ineffective organism by D-leucylglycine provides an interesting possibility for further research.

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THE UTILIZATION OF PURINES, PYRIMIDINES, AND INORGANIC NITROGENOUS COMPOUNDS BY EFFECTIVE AND INEFFECTIVE STRAINS OF *RHIZOBIUM MELILOTI*[1]

By D. C. Jordan and C. L. San Clemente

Abstract

Ammonium chloride was not utilized by three strains of *Rhizobium meliloti* as the sole source of nitrogen in a sucrose medium, unless either amino or certain non-nitrogenous carboxylic acids were also present. This was also essentially true for the utilization of nitrate, nitrite, purines, and pyrimidines, all of which are potentially able to form ammonia. These results may be interpreted on the assumption that washed cells of alfalfa – sweet clover rhizobia require, for growth initiation in a nitrogen-free medium, either preformed amino acids or compounds such as ammonia and certain carboxylic acids from which amino acids can be synthesized. Since α-ketoglutarate was extremely active in promoting growth in a medium containing ammonium chloride it was implied that the ammonia may be fixed by L-glutamic acid dehydrogenase activity, especially since this particular enzyme was located in these organisms. No aspartase activity could be demonstrated. The ineffective strain differed from the effective strains in that it was unable to use purines or pyrimidines as accessory nitrogen sources in amino acid media. This was a result of strain variation and it was not coupled with the state of ineffectiveness itself. A synthetic medium has been formulated for further growth studies on washed *Rhizobium* cells and for investigations on auxotrophic mutants of these bacteria.

Introduction

The literature dealing with the utilization of organic and inorganic nitrogenous compounds by alfalfa – sweet clover rhizobia is somewhat confusing, since various workers (2, 7, 10, 3) disagree as to the availability of many of these substances for growth purposes. These irregularities may be a result of strain variation or of differences inherent in the experimental conditions employed. In several cases the basal medium used contained complex materials whose exact composition was unknown and, in addition, little or no attention was directed toward the use of washed cells. In a more critical survey (4) it was found that strains of *Rhizobium meliloti* were capable of utilizing amino acids as sole sources of nitrogen and that these compounds apparently could not be replaced by ammonium chloride, even in the presence of a number of vitamins and nucleic acid fractions. Richardson (8), using the same techniques, was unable to report any utilization of either ammonium, nitrate, or nitrite salts or of any one of a large number of organic nitrogenous compounds, other than amino acids and their amides. The possibility exists, however, that many nitrogen-containing substances might be used by these bacteria if certain additional compounds were simultaneously present in the growth medium. The present investigation deals with the utilization of certain nitrogen sources in a purified basal medium containing amino or other

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Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ontario, and the Department of Microbiology, Michigan State University, East Lansing, Michigan. Part of a thesis submitted by the senior author to Michigan State University in May, 1955, in partial fulfillment of the requirements for the degree Doctor of Philosophy.

carboxylic acids as supplements. This represented an effort to clarify the situation in regard to the nitrogen nutrition of these microorganisms and to obtain data on possible metabolic differences among strains which were effective or ineffective in the fixation of molecular nitrogen.

Methods

The nitrogen-free basal medium, *Rhizobium meliloti* strains, and many of the general techniques employed have been previously described (5). The bacterial strains consisted of two (R_{21} and $R_{20\text{-}27A}$) which were efficient in symbiotic nitrogen-fixation and one (R_{20}) which could be termed ineffective or parasitic, depending on the host plant.

Sterile cuvettes were prepared which contained 9.0 ml. of pH 7.0 sterile basal medium, 0.5 ml. of sterile L-leucine solution (final concentration = 0.0001 *M*), and 0.5 ml. of a stock solution of either sodium nitrate, sodium nitrite, or ammonium chloride (yielding final salt concentrations equivalent to 0.01, 0.001, and 0.0001 moles per liter). The salt solutions were prepared from C.P. grade compounds and sterile double-distilled water and were sterilized by filtration. Similar cuvettes contained, in place of the salts, purines or pyrimidines* at a concentration of 0.0001 *M*. In another series of experiments tubes were employed which contained basal medium, ammonium chloride solution (final concentration = 0.01 *M*) or an equivalent amount of distilled water, and, substituting for the leucine, either a di- or a tri-carboxylic acid (0.025 *M* final concentration). The various tubes were inoculated with 0.2 ml. of a suspension of washed *Rhizobium* cells which contained 0.1 mgm. of bacterial nitrogen per ml. Incubation was carried out at 30° C., using basal medium controls which were either inoculated or uninoculated. Daily readings were taken with a Coleman photonephelometer until maximum nephelos values were obtained.

Attempts were made to replace the carboxylic acids by gaseous carbon dioxide. Basal medium of pH 7.5, containing 0.01 *M* ammonium chloride, was inoculated with washed rhizobia and incubated with suitable controls at 30° C. under carbon dioxide concentrations ranging from 5 to 10%. Daily observations were made for any evidence of growth.

Since all of the nitrogen compounds tested can potentially give rise to ammonia, and since the major pathways of bacterial amino acid synthesis from ammonia probably involve such activities as that of L-glutamic acid dehydrogenase and aspartase (11), it was believed advisable to detect the presence or absence of these two enzymes in rhizobia. For the study of dehydrogenase activity the conventional Thunberg technique was used, with methylene blue as the terminal hydrogen acceptor. The tubes contained 3 ml. of pH 8.0 *M*/40 phosphate buffer, 3 ml. of *M*/50 neutralized L-glutamic acid, 1 ml. of 1 : 10,000 aqueous methylene blue, and, in the cap, 1 ml. of washed cell suspension. The cells were obtained from an inoculated yeast-extract mineral-salts medium which had been incubated under aeration for

* *Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.*

72 hr. at 27° C. These cells, after being centrifuged, were washed twice in 0.85% saline, resuspended in 0.2 ml. of saline, and held at 5° C. for three days. This procedure ensured a low endogenous reduction of the dye. Just prior to use the cells were diluted with pH 8.0 *M*/40 phosphate buffer and standardized in a spectrophotometer to 18% T at 420 mμ, using the same buffer as a diluent. Endogenous tubes contained water in place of substrate and control tubes contained cells killed by boiling or inactivated by the additional of chlorobutanol. The reaction was studied at 42° C. by following the rate of dye reduction at 540 mμ (9).

Attempts to detect aspartase activity involved the anaerobic incubation of cell-free extracts (1.23 mgm. nitrogen per ml.) or acetone-dried cells (5.27 mgm. nitrogen per ml.) at 30° C., in the presence of pH 8.0 *M*/20 phosphate buffer, 0.5 *M* sodium fumarate, and 0.125 *M* ammonium chloride. In several cases cyclohexanol was added to inhibit any possible action of fumarase. After one hour the reaction was stopped by immersion in boiling water for five minutes, the mixture centrifuged, and 0.03 ml. samples of the supernatant analyzed for amino acids by paper chromatography in water-saturated phenol (14). Controls included cell-free extract alone, known amino acids, complete mixtures sampled at zero time, and a mixture from which the ammonium chloride (or fumarate) was omitted.

Results

In basal medium containing leucine the nitrate, nitrite, and ammonium salts were readily utilized as accessory sources of nitrogen by all three test organisms (Table I). Similar results were observed with the purines and pyrimidines, except in the case of the ineffective *Rhizobium* strain, where the growth was partially inhibited by these compounds (Fig. 1). No growth occurred with any of these compounds in the absence of leucine, but the

TABLE I

MAXIMUM GROWTH (IN NEPHELOS UNITS) ATTAINED BY THREE *Rhizobium* STRAINS IN LEUCINE MEDIUM CONTAINING INORGANIC NITROGENOUS COMPOUNDS

Strain	Molarity	Compound		
		NH_4Cl	$NaNO_3$	$NaNO_2$
R_{21} (effective)	0.01	570.0	455.0	5.0
	0.001	487.4	322.0	350.0
	0.0001	283.0	310.0	260.0
	0.0	174.0	174.0	169.0
$R_{20\text{-}27A}$ (effective)	0.01	552.0	414.0	4.0
	0.001	537.0	398.0	258.0
	0.0001	203.0	252.0	210.0
	0.0	114.0	116.0	120.0
R_{20} (ineffective)	0.01	532.0	350.0	4.0
	0.001	460.0	236.0	258.0
	0.0001	209.4	180.0	210.0
	0.0	116.0	116.0	120.0

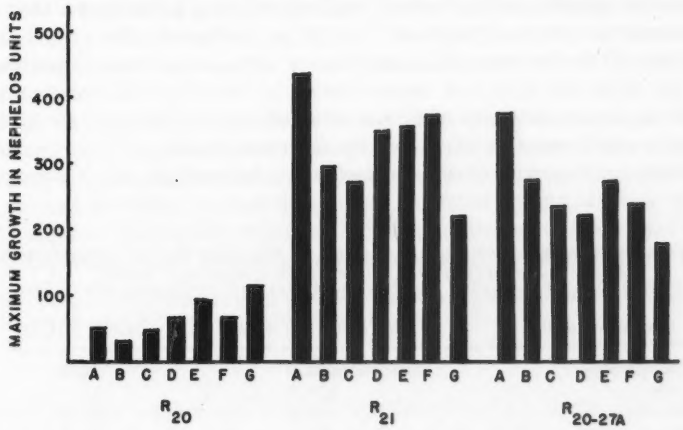

FIG. 1. Vertical bar chart showing the effect of certain purines and pyrimidines on the growth of three *Rhizobium* strains in leucine basal medium. (*A*) adenine sulphate, (*B*) guanine HCl, (*C*) thymine, (*D*) uracil, (*E*) cytosine, (*F*) xanthine, (*G*) leucine base only.

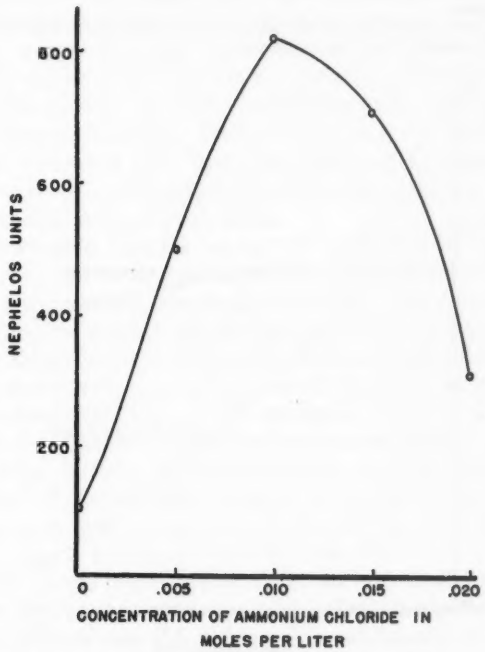

FIG. 2. Growth-response curve of *Rhizobium* R_{21} in basal medium containing 0.0001 *M* histidine and various concentrations of ammonium chloride.

activity is not specific in that subsequent experiments have shown that this substance can be effectively replaced by a large number of other amino acids. The best growth on the ammonium and nitrate salts occured at a concentration of 0.01 *M*, while the optimum concentration of nitrite was about 0.001 *M*. The lower optimum molarity of nitrite is a reflection of the greater toxicity of this salt, which may be explained by the consideration that it combines with the free amino groups of cellular proteins. Ammonium chloride was very

TABLE II

MAXIMUM GROWTH (IN NEPHELOS UNITS) ATTAINED BY *Rhizobium* R_{21} IN A SYNTHETIC BASAL MEDIUM CONTAINING NON-NITROGENOUS CARBOXYLIC ACIDS

Carboxylic acid*	Plus NH_4Cl (0.01 *M*)	Minus NH_4Cl†
α-Ketoglutarate‡	204.0	24.0
Pyruvate	158.0	15.0
Malate	140.8	18.0
Citrate	128.5	24.0
Fumarate	116.3	13.0
None (basal medium only)	0.0	0.0

** Acetate and succinate were inactive at the concentration used.*

† This growth may have developed at the expense of trace amounts of combined nitrogen present in the medium constituents.

‡ α-Ketoglutarate plus either 0.01 M nitrate, 0.001 M nitrite, or 0.0001 M adenine yielded values of 165.1, 126.3, and 72.8 units respectively.

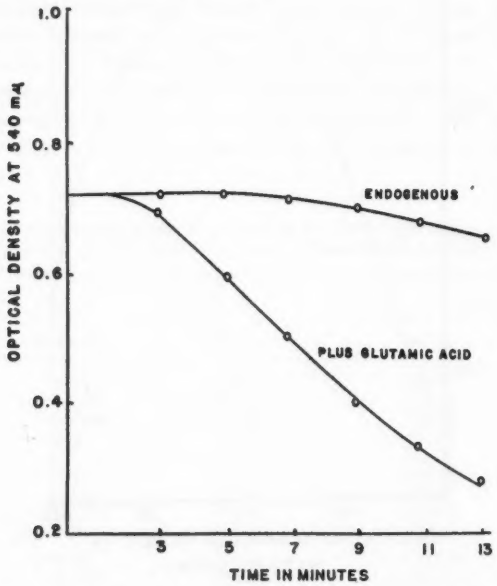

FIG. 3. The anaerobic reduction of methylene blue by *Rhizobium* R_{21} in the presence and absence of L-glutamic acid.

effective in promoting growth and the growth-response curve of this compound in histidine medium is shown in Fig. 2. Histidine is a very excellent nitrogen source for *Rhizobium meliloti* (5) and exerts its greatest effect at 0.0001 *M* concentration (8).

Table II lists values which indicated that the amino acid in the basal medium could be effectively replaced by at least several non-nitrogenous carboxylic acids plus ammonium chloride. These carboxylic acids could not be replaced by added carbon dioxide, as indicated by the absence of growth in the ammonium chloride tubes incubated under increased tensions of this gas.

An L-glutamic acid dehydrogenase was located in these rhizobia (Fig. 3), but, under the experimental conditions used, there was no evidence for the synthesis of any amino acid via aspartase activity.

Discussion

The fact that ammonium chloride was found to be a better nitrogen source than either nitrate or nitrite probably emphasizes the prime importance of ammonia, especially since the nitrate, nitrite, purines, and pyrimidines are all capable of producing this substance during metabolism. The observed results may, therefore, be interpreted by assuming that "resting" *Rhizobium* cells require a source of amino acids for growth initiation and that these compounds may be obtained intact from the medium or synthesized from ammonia and one or more non-nitrogenous carboxylic acids. These growth-promoting acids probably do not accumulate intracellularly in resting cells in quantities compatible with growth on media containing only ammonia-forming compounds as nitrogen sources. These results are not entirely novel since Virtanen and Rautanen (12) have reported that Das observed growth of *Aerobacter aerogenes* on ammonium nitrogen in the presence of several organic acids of importance in the Kreb's cycle.

Some heterotrophic bacteria are known to require the presence of added carbon dioxide for optimum growth and the substitution of several dicarboxylic acids for this gas can satisfy the requirement (1). The effect of the carboxylic acids on the rhizobia was not due, apparently, to a carbon dioxide requirement as this gas could not replace these acids in ammonium ion utilization.

One of the more prominent compounds in the bacterial synthesis of amino acids is α-ketoglutarate, from which substance glutamic acid can be formed by a reversal of L-glutamic acid dehydrogenase activity. This reaction may explain the high activity of α-ketoglutarate in the present investigation, especially since the responsible enzyme is present. The other carboxylic acids may or may not be reactive in themselves, but would probably be capable of forming α-ketoglutarate within the cell.

The inability of the ineffective strain to use the nucleic acid fractions is of some interest and merits further research, although unpublished data by the senior author indicate that this deficiency is not directly related to the condition of ineffectiveness itself. Indeed, the ability to utilize nitrogenous compounds does not appear too promising as a biological indicator of nitrogen-fixing ability.

The data accumulated in this and other investigations have resulted in the production of an adequate synthetic medium for growth studies on washed cells of *Rhizobium meliloti*. It consists essentially of the purified medium of Wilson and Wilson (13), as modified in the present work, with several supplements. It is composed of sucrose, 2.50 gm.; ammonium chloride, 0.53 gm.; dipotassium hydrogen phosphate, 0.50 gm.; sodium chloride, 0.10 gm.; magnesium sulphate, 0.20 gm.; calcium sulphate, 0.20 gm.; ferric chloride, 0.10 mgm.; histidine, 7.50 mgm.; glass-distilled water, 1000 ml. The pH is adjusted to 7.0 or 7.5 and sterilization accomplished by Seitz filtration. If so desired, the histidine may be replaced by 0.025 *M* α-ketoglutarate. This modification adapts the solution for use as a basal medium for the study of auxotrophic mutations involving amino acid requirements.

Several workers (3, 6) regard nitrite as being non-utilizable by the alfalfa – sweet clover rhizobia. Indeed, the inability to make use of nitrite is one of the criteria of the genus *Rhizobium*, although Pohlman (7) found that there was some indication of a slight utilization by several species. Obviously, nitrite can be used provided that certain conditions are fulfilled. It is suggested, therefore, that the statement concerning the action of rhizobia on nitrite, as given in Bergey's Manual of Determinative Bacteriology, be modified in subsequent editions.

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ON SOME PARASITES OF PARASITIC PROTOZOA

II. *SAGITTOSPORA CAMERONI* GEN. N., SP. N.—A PHYCOMYCETE PARASITIZING OPHRYOSCOLECIDAE[1]

By G. Lubinsky

Abstract

A new genus and species of chytrid fungi, parasitizing Ophryoscolecidae, is described. Its spores are similar to the "peg-formed organisms" of Kirby. It is pointed out that some hyperparasitic microorganisms, hitherto classified as Microsporidia, but lacking essential characters of this order, are probably chytrid fungi, related to *Sagittospora cameroni* gen. et sp. nova.

Introduction

After the discovery of chytrid fungi parasitizing lower plants and protozoa a trend appeared to designate loosely as "chytrids" any fungus-like parasite of uncertain affinities found in the above hosts. The parasites of Ophryoscolecidae have shared in this common fate: genuine chytrids, parasitizing these ciliates, were confounded with spherical accumulations of bacteria and with divers other microorganisms and appeared in the literature under the name "Sphaerita"—originally a name of a genus of Olpidiaceae, which, however, in protozoological literature practically became a collective name for any spherical parasites found in protozoa. The paper of Winogradowa (9) on the parasites of Ophryoscolecidae is no exception to this rule: in a brief note—2 pages—she describes as "Sphaerita" divers microorganisms without giving any evidence of them being Chytridiales. Organisms described by her as "third form" of *Sphaerita* deserve special attention. These parasites, found by Winogradowa in a ciliate of the genus *Eudiplodinium* Dogiel (a genus subsequently divided by Kofoid and MacLennan into the genera *Eudiplodinium* s. str., *Diplodinium*, and *Eremoplastron*), are depicted in her Fig. 6 (reproduced by Kirby (5), p. 1049), and described in the following words (translated from the German):

"The parasites of this form are arranged in the cytoplasm of the ciliate in rosettes each containing 8 specimens of ovoidal or boat-shaped parasites. The number of rosettes in one ciliate reaches 50–60. The size of each rosette is 10–12μ [in the original: 10–12 mm.—an obvious misprint. Author], the individual parasites being 5–6μ long and 2–3μ wide."

This peculiar arrangement of what is called "individual parasites" certainly does not fit into the morphological characteristics of the genus *Sphaerita*. Parasites of Ophryoscolecidae, probably identical to the "third form of Sphaerita" of Winogradowa, were found in this Institute in the course of an investigation of the fauna of ophryoscolecid ciliates of ruminants. The present paper is an attempt to describe these parasites and to discuss their systematic position.

[1] *Manuscript received May 10, 1955.*
Contribution from the Institute of Parasitology, McGill University, Macdonald College, Quebec, Canada, with financial assistance from the National Research Council of Canada.

Materials and Methods

The faunule of the Jamnapari goat with infected specimens of *Eudiplodinium maggii*, on which the description of the type-specimen is based, was collected by Lt. Mohammad Shafique, Veterinary Surgeon, R.P.A.S.C. in Quetta, W. Pakistan, in December 1951. Other faunules with infected ciliates from sheep and goats were collected by me in 1951 in Rawalpindi-Cantt. The material was fixed and kept in 10% formalin. Thick films were prepared using methods described in a previous paper (8). Mayer's acid hemalum in Hoare's modification, Heidenhain's hematoxylin, and Gower's carmine were used for staining.

Results

The faunule of ciliates from the rumen of goat G.Q.1 (goat 1 from Quetta) contained several species of *Entodinium* and five of higher Ophryoscolecidae: *Diplodinium minor* Dogiel 1925, *Eudiplodinium maggii* (Fiorentini 1889), *Eremoplastron bovis* (Dogiel 1927), *Metadinium tauricum* (Dogiel and Fedorowa 1925), and *Epidinium caudatum* (Fiorentini 1889). Only two of these species were found to be infected with the parasite in question: *Diplodinium minor** and *Eudiplodinium maggii*. In both, the infection rate was low—1.2 and 2.1% respectively. Most of the infected specimens contained what Winogradowa called rosettes of "individuals". Closer examination revealed that these were spores produced by a parasitic fungus.

The earliest stage of the parasite found in our material was a plasmodial thallus situated in the endoplasm of the ciliate and frequently invading the ectoplasm. It is hard to distinguish the cytoplasm of the parasite from that of the host. Only in few infected specimens of both *D. minor* and *E. maggii* could the boundaries of the plasmodium be clearly seen (Figs. 6, 9). The cytoplasm of the plasmodium is not differentiated into endoplasm and ectoplasm. It is vacuolated, finely granular, and contains numerous rounded nuclei 2–5 μ in diameter. Dividing nuclei are spindle-shaped (Fig. 9). When the plasmodium is entirely within the endoplasm of the host, its outlines are indistinct and the nuclei may be mistaken for ingested food particles. However, as soon as the ectoplasm of the ciliate becomes invaded, numerous nuclei appear in it. In some hosts the ectoplasm of the adoral lips and the operculum was found to be replaced by the plasmodium of the parasite and to contain numerous nuclei.

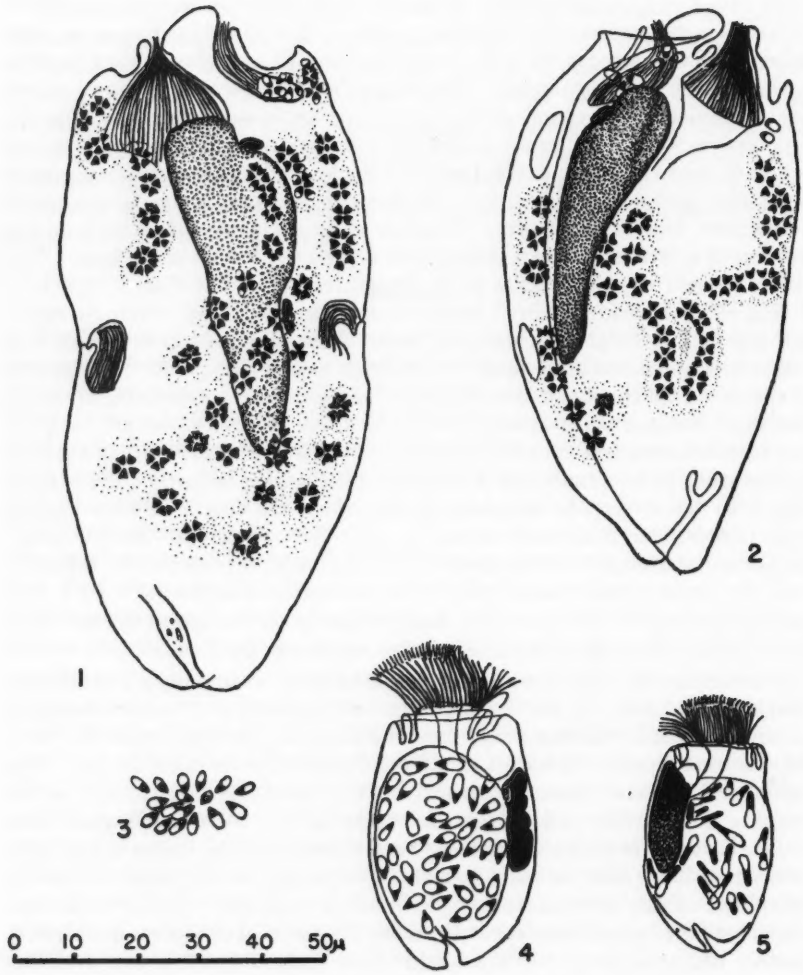
When the thallus has filled nearly the whole body of the host, spore formation sets in. Nuclei, previously scattered in the plasmodium, accumulate in numerous centers—the future places of spore formation, usually arranged in longitudinal rows (Figs. 1 and 2). The nuclei of the future spores assume a conical shape and converge with their apices towards the center of the group

* *The shape of the macronucleus in this species exhibited continuous variation which included macronuclei typical for both D. minor Dogiel 1925 (Anoplodinium costatum forma minor Dogiel 1925) and D. dogieli Kofoid and MacLennan 1932 (Anoplodinium rangiferi forma minor Dogiel 1925).*

(Fig. 10). Numerous rounded nuclei still remain among these groups in the early stages of spore formation. Near the peripheral blunt end of the conical nuclei of spore-rudiments, spherical portions of a more hyaline cytoplasm appear. The spore-nuclei with these portions of cytoplasm soon become surrounded by a membrane. The formation of spore-membranes occurs simultaneously in all spores of a given group; spore-delimitation is thus not progressive. The ripe spore is ovoidal. Its pointed end, directed to the center of the spore group, is filled with the nucleus, while the clear homogenous cytoplasm occupies its wide end. The number of spores formed in one center varies from four to about fifty. The size of spores is dependent both on the number of spores formed in a given center and on the species of the host. The spores formed by the parasite in *E. maggii* measured $4(3\text{–}7)\mu \times 1.6(1.4\text{–}2.0)\mu$, while those formed in *D. minor*—a much smaller ciliate—were shorter—$3(2.5\text{–}4.0)\mu \times 1.5(1.4\text{–}1.6)\mu$. The larger the number of spores formed in a center in a given host, the smaller the average size of the spores. The groups of spores are spherical or ellipsoidal, rarely elongate, the spores being arranged radially. Around these groups and inside them the cytoplasm of the plasmodium becomes less stainable with hemalum with the result that the ripe spores seem to be situated in a kind of vacuole with indistinct boundaries. The clear halos, seen in hemalum-stained films, are absent in films stained with Gower's carmine, which does not stain the cytoplasm. As the spore-formation progresses, the cytoplasm of the plasmodium progressively liquefies, and the regular radial arrangement of the spores disappears. In a ripe sporangium, which fills the entire body of the host, the spores are arranged irregularly. No trace of their polycentric origin can be found.

The nuclei of the ripe spores—the "arrow-heads"—are readily stainable with Mayer's acid hemalum, Heidenhain's iron hematoxylin, and Gower's carmine. In slides, stained with hemalum, these nuclei are as dark as, or darker than, the macronucleus of the host, and always darker than its micronucleus. The cytoplasmic part of spores is stained by this stain but faintly, and so are bacteria both within and outside the bodies of ciliates. Iron hematoxylin stains the nuclei of plasmodia and the "arrow-heads" of the spores intensively. Prolonged differentiation with iron alum, necessary to decolorize the bulky cytoplasm of the host-ciliates, leaves bacteria colorless. Gower's carmine, which is a fairly specific nuclear stain, stains the nuclei of the spores intensively, leaving the cytoplasm unstained. In films stained by this last method, bacteria were always colorless and nearly invisible.

The number of centers of spore-formation varies between 50 and 200 depending on the size of the host. It is much larger in *E. maggii* than in *D. minor*. The anterior end of the ciliate usually contains more spore-formation centers than the posterior. It is probable that the infection usually starts near the anterior end of the host—and it may be that the spores of the parasites are ingested by the ciliate. We have not found in our material specimens in the process of discharging spores, and have not seen the formation of discharge-papillae, although we have examined over a hundred infected



FIGS. 1 and 2. *Diplodinium minor* infected with *Sagittospora cameroni*. The ciliate shown in Fig. 1 is dividing.

FIG. 3. Group of spores of *S. cameroni* outside the body of the host.

FIG. 4. Spores, similar to those of *S. cameroni*, in the endoplasm of an *Entodinium* of the "dubardi" type.

FIG. 5. Club-shaped spores in *Entodinium* sp.

FIGS. 1-5. Magnification × 960.

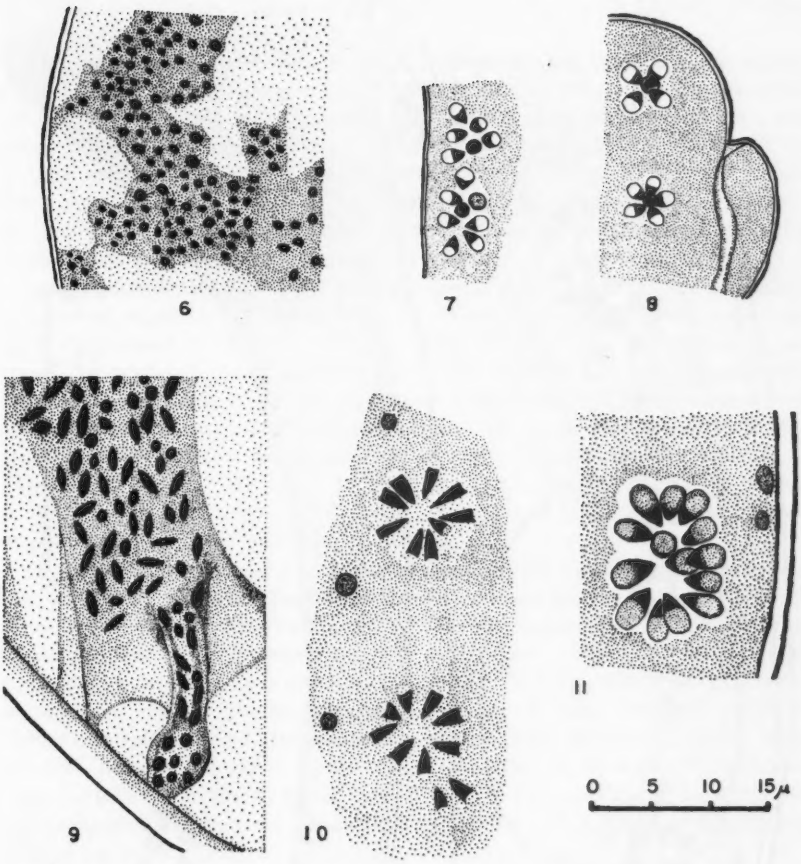


FIG. 6. Plasmodial thallus of *S. cameroni* in *Diplodinium minor.*

FIGS. 7 and 8. Spore-formation centers of *S. cameroni* in *Diplodinium minor.*

FIG. 9. Plasmodial thallus of *S. cameroni* in *Eudiplodinium maggii.* Note the spindle-shaped dividing nuclei.

FIG. 10. Early stage of spore-formation of *S. cameroni* parasitizing *E. maggii.*

FIG. 11. Late stage of spore-formation of *S. cameroni* in the same host.

FIGS. 6-11. Magnification × 1833.

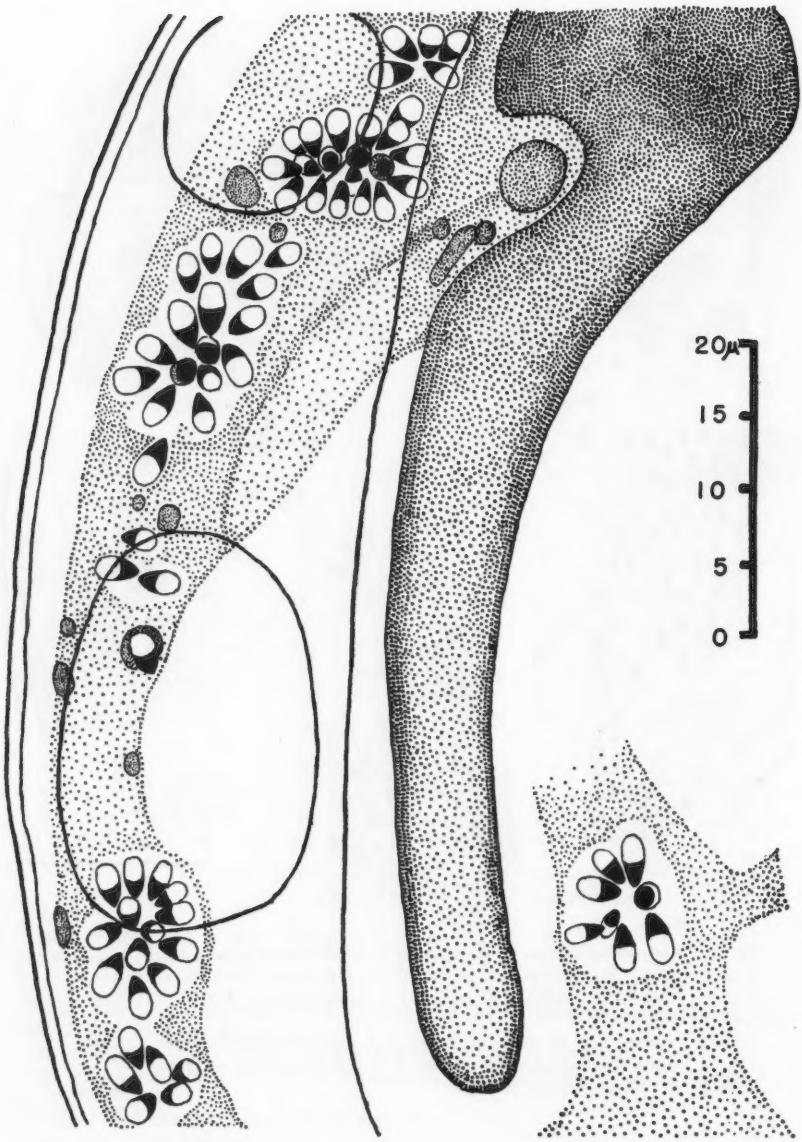


FIG. 12. Type specimen of *Sagittospora cameroni* in *Eudiplodinium maggii*. (Slide G.Q.1/28.) Magnification × 2200.

ciliates. But free groups of spores outside the body of the host were found several times. These groups consisted of 10–30 spores, with their pointed ends directed towards the middle of the group (Fig. 3). The individual spores seemed to be embedded in a transparent jelly, holding them together. Flagella have not been seen.

The parasite invariably kills its host. However, the thalli and the unripe sporangia are very well tolerated. One is astonished to see the infected ciliates dividing despite the presence of a gigantic plasmodium, already in the process of spore-formation (Fig. 1). In such specimens of ciliates the structure of both macronucleus and micronucleus is normal. But as soon as the spores are ripe and evenly distributed in the sporangium (which nearly fills the entire body of the ciliate without however invading the nuclear apparatus) both the macronucleus and micronucleus become pycnotic and the host dies.

In the faunule of goat G.Q.1 we have found a few specimens of *E. maggii* containing single large spherical bodies 30–40μ in diameter with a smooth membrane about 0.8μ thick. They contained numerous irregularly arranged spore-like bodies 1.5μ in diameter. These spheres, although superficially resembling sporangia of *Sphaerita*, differed from these last in the irregularity of the arrangement of the spore-like bodies and in the absence of an endosporium. The spheres are reminiscent of resting spores of Chytridiales and Plasmodiophorales. We have no proof that they are resting-spores of our fungus, but their occurrence in the same faunule and in the same host-species as our fungus suggests such a possibility.

Organisms, morphologically indistinguishable from our fungus, were found also in another faunule, that of the Jamnapari goat G68, collected in Rawalpindi in July 1951. Only two infested ciliates—one specimen of *Diploplastron affine* and one of *Elytroplaston bubali*—were seen. The infected *D. affine* contained numerous spores measuring about 3.0 × 1.5μ, which formed three-dimensional stellate groups, the individual spores being arranged radially. Numerous groups filled the endoplasm of the host, but a few spores were situated in the ectoplasm of the outer dorsal lip and in that of the caudal lobes.

In the infected *E. bubali* the spores—3.0–4.2μ long and about 1.5μ wide—were scattered throughout the cytoplasm but formed a dense accumulation around the macronucleus.

Spores, similar to those of our fungus, were also found in one specimen of *Entodinium* sp. in another faunule—that of sheep 54 from Rawalpindi. The irregularly arranged ovoidal spores, measuring about 4 × 2μ, filled the entire endoplasm of the ciliate. The macronucleus of the host was pycnotic (Fig. 4). Despite a thorough search, no other infected ciliates were found in this faunule.

In still another faunule—that of Jamnapari goat 67 from Rawalpindi—one specimen of *Entodinium* sp. containing club-shaped spores measuring about 7 × 1.5μ was found (Fig. 5). The origin of the spores could not be investigated owing to the scarcity of infected ciliates.

The shape of spores, found in the cytoplasm of *Entodinium* sp. in these two faunules, resembled superficially that of bacterial spores. But the narrow

ends of these spores were stained with hemalum as deeply as the macronuclei of their hosts, whereas the bulky cytoplasm of *Entodinium* and that of bacteria, scattered among the ciliates, remained nearly colorless. The above spores were found only inside the cytoplasm of *Entodinium* and were not seen elsewhere. Because of the scarceness of infected ciliates the origin of these spores could not be investigated. Their relation to our fungus remains therefore unclear.

Discussion

The spores of the fungus described are similar both in shape and size to the spores of some Microsporidia. However, Microsporidia, usually classified as Protozoa, produce multicellular spores possessing a polar filament, capsule-producing cells, and an ameboid body. But the spores of our fungus are unicellular and resemble spores of some Phycomycetes as, for example, *Catenaria* and *Coelomomyces*, being, however, aflagellate. The presence of an endobiotic holocarpic thallus without any evidence of karyological processes characteristic of Plasmodiophorales compels us to place our fungus provisionally in the order Chytridiales. We can exclude the operculate series, as well as families of the inoperculate series with eucarpic thalli. Our fungus can not be placed in the families Achlyogetonaceae or Synchytriaceae characterized by formation of several sporangia. The representatives of the remaining family—the Olpidiaceae—produce only one sporangium. The infestation with our fungus terminates in the transformation of the whole host-body into a sac filled with spores. But such a "sporangium" lacks proper walls. However in the Olpidiaceae we find one genus—*Rozella*—which forms sporangia with walls fused with those of the host cell. Moreover *Rozella*, as well as *Plasmophagus*, has plasmodial thalli. Our fungus does not fit into these genera, which have flagellate spores, although we can provisionally place it in the family Olpidiaceae. As it differs considerably from all other genera of this family in the structure of its spores and in the absence of the wall of sporangium we propose to create for it a new genus, with the type species *Sagittospora cameroni* in honor of Dr. T. W. M. Cameron, F.R.S.C., of McGill University. The generic name refers to the likeness of the spore-nuclei in side-view to arrow-heads.

Diagnosis

Sagittospora gen. n.

Type species: ***Sagittospora cameroni*** sp. n.

Thallus holocarpic, endobiotic, plasmodial, naked, with numerous nuclei 2–5μ in diameter. Sporangium naked, filling the entire body of the host. Spores formed in numerous centers producing each from 4 to 60 ovoidal spores 3–7μ long and about 1.5μ wide. Nucleus filling the narrow end of the spore in close contact with the spore-membrane.

Type host: *Eudiplodinium maggii* from the rumen of a Jamnapari goat.

Type locality; Quetta, W. Pakistan. Collected in December 1951.

Type specimens are preserved in the Institute of Parasitology, Macdonald College, P.Q.

Sagittospora genus novum

Species typica: ***Sagittospora cameroni*** sp. n.

Thallus holocarpicus, endobioticus, plasmodialis, nudus, cum nuclei numerosi 2–5μ diametro. Sporangium nudum, corpus hospitis complens. Sporae in sedibus numerosis productae sunt, quarum quaeque sedes 4–60 sporas ovoidales 3–7μ longas, circa 1.5μ latas, parit. Nucleus terminum acutum sporae complens, ad membranam sporae propinquiter applicatus est.

Hospes typicus: *Eudiplodinium maggii* ex ruminis caprae (Capra hircus, Jamnapari capra).

Locus typicus: Quetta, Pakistan occidentalis; Coll. December, 1951.

Similar parasites, found in the same faunule but parasitizing *Diplodinium minor*, differ from the above-described typical form in the smaller number of spores formed in one center (4–20 as compared to 4–60 in the typical form) and in the shortness of the spores (2.5–4.0μ as compared with 3–7μ of the typical form). These differences are probably hostal: *Sagittospora* is a rare parasite found in less than 1% of faunules examined and the probability of finding two species of this genus in one faunule is low. However, as experimental proof of the genotypical identity of these two forms is lacking, it is preferable to restrict the description of the type species to forms found in one host only.

It is improbable that *S. cameroni* is a unique species of a monotypical genus. In the literature can be found several references to similar organisms, which are, noteworthy enough, always hyperparasites.

The similarity between the spores of *S. cameroni* and the "peg-formed organisms" found by Kirby in *Stephanonympha* (but not in *Trichonympha* (4)) is striking (see (5), p. 1031, Fig. 216A). Kirby has not investigated the life-cycle of these organisms, but has discussed them under the heading "Schyzomycetes in Mastigophora". It is remarkable that both *S. cameroni* and the "peg-formed organisms" are found parasitizing cellulose-digesting intestinal protozoa: the first, the Ophryoscolecidae of ruminants; the second, the polymastigote flagellates of termites. It is probable that the "peg-formed organisms" found in *Stephanonympha* by Kirby are spores of a fungus related to *Sagittospora*.

Spores of another hyperparasite—*Nosema legeri* Dollfus 1912—are also extremely similar to the spores of *Sagittospora* (1, 2). The organism, subsequently named by Dollfus *Nosema legeri*, was first found by Giard (3) already in 1897 infesting a metacercarium parasitizing a marine pelecypod mollusc. Leger (7), who studied this hyperparasite, stated that the spores "de forme ovoïde renflée à une extrémité qui montre une vacuole très nette et legèrement arroundie à l'autre" measure about 5 × 2.5μ and are all of approximately equal size. In 1912 Dollfus has described this parasite under the name "*Nosema legeri*". His drawings, made at a magnification of 1435, show numerous ovoidal spores of variable size measuring 2.2–3.1 × 1.1–1.7μ—

these dimensions coincide fairly well with those of spores of *S. cameroni* (1, 2). Guenot and Naville, who re-examined *N. legeri* from the type locality, placed this organism in the genus *Plistophora*, characterized by a variable number of spores produced in one sporoblast. Kudo (6) stated that "Polar capsule, filament and valve-nuclei were never noted" in *N. legeri*. It is possible, therefore, that this organism is not a microsporidian, but a fungus. It has to be noted that spores of many organisms, now classified as Microsporidia, are extremely similar to spores of chytrid fungi. The presence of a flagellum, which can be mistaken for an extruded polar filament of which only the proximal part was stained, may add to the confusion. Moreover, sporoblasts and "secondary cylinders" of many species of Microsporidia are suspiciously similar to thalli of Chytridiales. It is probable, therefore, that some of the Microsporidia will be found to be fungi, as has already been shown to be the case with several genera of Haplosporidia.

There exist thus several microorganisms similar to our fungus; *S. cameroni* is not a unique organism, but a representative of a group of organisms found parasitizing endozoic protozoa of both mammals and insects and also marine trematodes. What is the taxonomic value of this group? Our present-day knowledge of these peculiar hyperparasites is not sufficient to state whether it is merely a genus or a group of higher taxonomic rank.

Acknowledgment

The author wishes to express his thanks to Lt. Mohammad Shafique, Veterinary Surgeon, R.P.A.S.C., for the kindess of collecting rumen contents of domestic ruminants in Quetta in 1951.

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ASSAY FOR CIRCULIN BASED ON THE RELEASE OF CELLULAR CONSTITUENTS[1]

By D. J. Colasito[2], Henry Koffler[3], P. A. Tetrault[3], and H. C. Reitz[4]

Abstract

Circulin causes the release of cellular components from susceptible cells exposed to its action; within a given range of circulin concentrations this release is proportional to the amount of circulin present. This relationship can be used as a basis of a rapid and sensitive method for determining circulin. Inasmuch as cells on exposure to circulin release numerous compounds, various means can be used to measure this release, and the assay can be adapted to the equipment available. Two applications of this principle are described. They are based on the release from suspensions of *Escherichia coli* ATCC 26 of compounds that absorb radiations having a wave length of 260 mμ, or of P^{32}-containing compounds from cells grown in a medium containing radioactive phosphate. Basically the same procedure is used for both assays: The cells are exposed to the action of various concentrations of circulin in centrifugation tubes. After two hours the cells are centrifuged down, and an aliquot of the supernatant liquid is examined either for absorption at 260 mμ, or for radioactivity. Unknown amounts can be determined by a comparison of their effectiveness in causing release of cellular materials to that of known amounts. By adjusting the initial concentrations of cells, one can vary the range of circulin concentrations over which the assay can be used. This method is more sensitive and more rapid than the filter disk – agar diffusion method of assay.

Introduction

Circulin, a mixture of basic peptide antibiotics belonging to the polymyxin family, is assayed routinely by the filter disk – agar diffusion procedure of Stansly and Schlosser (16), as modified by Murray *et al.* (9). This procedure is sensitive to approximately 10 μgm. per ml. During a study on the mechanism of action of circulin it became necessary to develop a more sensitive and more rapid analytical method of assay for this antibiotic.

Several antibacterial compounds, such as cetyltrimethylammonium bromide (13, 14, 15), tyrocidin (6, 7, 8), subtilin (12), and polymyxin (4, 5, 10, 11), have been reported to cause the release of cellular materials into the surrounding medium. Inasmuch as a variety of protoplasmic constituents are released, several means can be used to detect this damage. For example, the appearance in the suspending fluid of the following has been shown: materials absorbing radiation at a wave length of 260 mμ (3, 4, 5, 11, 15); radioactive phosphorus from P^{32}-labelled cells (3, 14); nitrogen-containing compounds (7, 8, 12, 15); phosphorus-containing compounds (7, 8, 10, 12, 13, 15); specific amino acids (6, 15); pentoses (10, 15); and ninhydrin-positive materials (2).

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Since even minute amounts of the antibacterial agents previously mentioned are capable of causing measurable damage, it seemed likely that the relationship between the release of a given cell substance and the amount of compound necessary to cause this release could be used as a basis of a method for the quantative determination of these agents. This paper describes two procedures for the assay of circulin based on this relationship.

Materials and Methods

Escherichia coli ATCC 26 was used as the test organism. The circulin used was preparation No. 177-HAN-14 kindly furnished us by Drs. D. H. Peterson and D. R. Colingsworth of the Upjohn Company, which assayed 5300 units per mgm. and contained at least two major components (circulins *A* and *B*). Two criteria were used to determine the degree of cellular damage: (1) release of compounds absorbing radiations having a wave length of 260 mμ ("260 mμ absorbing materials"), and (2) release of P^{32}-labelled compounds from cells grown in a medium containing radioactive phosphate.

For the first procedure, cells were grown in a nutrient broth, having the following composition in grams per liter: Bacto-peptone, 5; Bacto-beef extract, 3; and sodium chloride, 5. For the second procedure the following medium was used (in grams per liter): trypticase, 17; glucose, 2.5; and sodium chloride, 5. $KH_2P^{32}O_4$ was added so that the medium contained radioactive phosphorus at a level of 10^7 counts per minute per milliliter of medium. Cultures were grown for 18 hr. at 37° C. in 500 ml. Erlenmeyer flasks containing 100 ml. of medium under agitation by a wrist-action shaker. Cells were harvested by centrifugation, and washed twice with *M*/15 Sørensen's phosphate buffer of pH 7.0. The cells were resuspended in this buffer; the density of the resulting suspension was determined by direct microscopic count with a Petroff-Hauser counting chamber, and then adjusted to desired levels. Aliquots of the cell suspension were added to centrifugation tubes containing various levels of circulin in the same buffer. The final volume in each tube was 10 ml. After being shaken to insure an even suspension, the tubes were incubated for two hours in a refrigerator (6°–8° C.). The tubes were then centrifuged for about 20 min. at 2000 × *g*, and the supernatant liquid was withdrawn and recentrifuged.

The amount of "260 mμ absorbing materials" in these supernatant solutions was determined by measuring their optical density at 260 mμ in the Beckman DU spectrophotometer. The amount of material released in the absence of circulin (zero level circulin) was used as the blank value. Radioactivity released from labelled cells was measured as follows: 1-ml. aliquots of the supernatant fluids were placed in stainless steel planchets, and dried under an infrared lamp. After the samples had dried, their radioactivity was measured with a Geiger-Müller counting tube. The release of label in the absence of circulin was used as blank. Because of the relatively short half-life of P^{32} it was impractical to obtain identical labelling of cells from one experiment to the next. Instead, the degree of release was expressed as "per cent total

radioactivity". The total amount of radioactivity in the cells was determined by the following procedure: 1-ml. aliquots of uniformly suspended cells were placed in planchets, and the cells hydrolyzed by the application of 1 ml. of 6 *N* HCl and heat. The samples were then dried and counted with the others. After the subtraction of background and normal release, all values were then expressed in terms of per cent of the total radioactivity contained in the cells at zero time.

Results

Effect of Time on Release of Cellular Constituents

To ascertain the length of incubation necessary for the maximum release of protoplasmic constituents in the presence of circulin, the appearance of these materials in the medium with time was determined. Cells were exposed to circulin levels of 1, 10, and 45 μgm. per ml., and samples were examined at intervals. As is shown in Fig. 1, the release of "260 mμ absorbing materials" has stopped after 60 min. The same holds true for the release of P^{32}-containing compounds (Fig. 2). A two hour period of incubation was adopted as standard practice for both procedures.

Reproducibility of Standard Curves

Fig. 3 is a standard curve obtained when the mean values of 10 assays employing the release of "260 mμ absorbing materials" are plotted; the assays, each in duplicate, were run on separate days. Included on this graph are the standard errors of the means. Table I lists the ranges of the 10 assay

TABLE I

REPRODUCIBILITY OF STANDARD CURVE WHEN RELEASE OF "260 mμ ABSORBING MATERIALS" WAS USED AS BASIS OF ASSAY

Circulin, μgm./ml.	Optical density at 260 mμ		
	Range for 10 runs	Mean	Standard error
0.5	.001–.007	.004	± .0040
1	.008–.016	.011	± .0015
5	.041–.053	.050	± .0019
10	.097–.119	.104	± .0031
20	.185–.201	.189	± .0039
30	.279–.310	.292	± .0057
40	.352–.372	.366	± .0068
45	.372–.398	.393	± .0070
50	.403–.425	.413	± .0063
60	.429–.436	.431	± .0078
75	.440–.451	.447	± .0121

NOTE: *Conditions as in Fig. 3.*

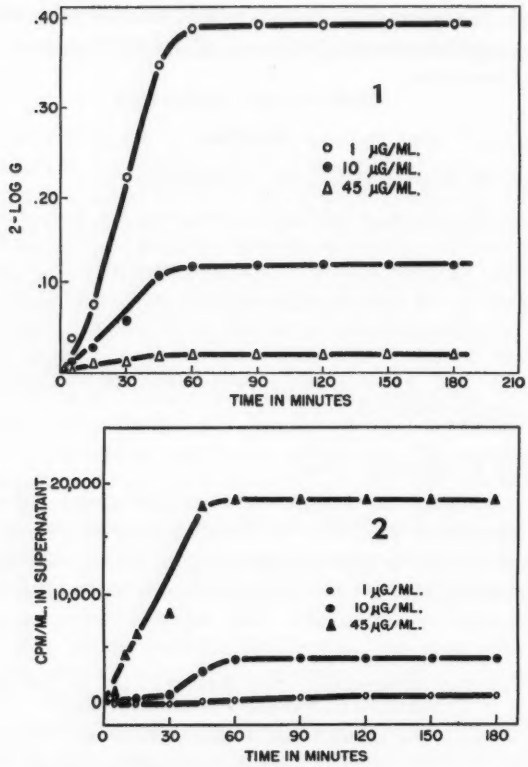

FIG. 1. Release of "260 mμ absorbing materials" from cells of *Escherichia coli* ATCC 26 with time caused by circulin at three concentrations. Cell density, 10^7 per ml.; temperature, 6°–8° C. Normal release of absorbing materials (zero level circulin) gave blank values that were subtracted from those obtained when circulin in various concentrations was present. At 120 min., for instance, the zero level circulin sample read .004 against a buffer control.

FIG. 2. Release of P^{32}-containing compounds from P^{32}-labelled cells of *Escherichia coli* ATCC 26 with time caused by circulin at three concentrations. Cell density, 10^7 per ml.; temperature, 6°–8° C. Normal release of radioactivity (zero level circulin) gave blank values that were subtracted from those obtained when circulin in various concentrations was present. At 120 min., for instance, the zero level circulin sample read 764 c.p.m. above background.

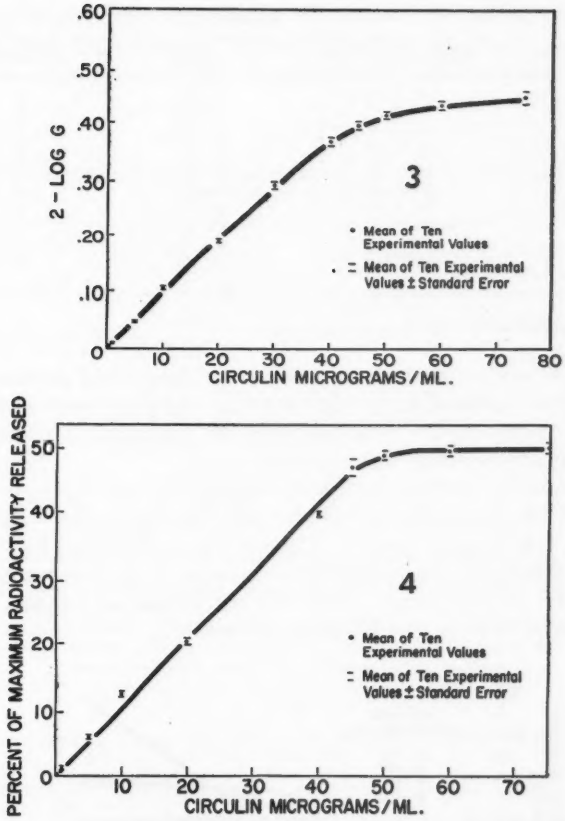

FIG. 3. Amount of "260 mμ absorbing materials" released from cells of *Escherichia coli* ATCC 26 (10^7 cells per ml.) after two hours of incubation (6°–8° C.) in the presence of various levels of circulin.

FIG. 4. Per cent of radioactivity released from P^{32}-labelled cells of *Escherichia coli* ATCC 26 (10^7 cells per ml.) after two hours incubation (6°–8° C.) in the presence of various levels of circulin.

TABLE II

REPRODUCIBILITY OF STANDARD CURVE WHEN RELEASE OF RADIOACTIVITY FROM P^{32}-LABELLED CELLS WAS USED AS BASIS OF ASSAY

Circulin, μgm./ml.	Per cent of total radioactivity released		
	Range for 10 runs	Mean	Standard error
0.5	0.3– 0.5	0.42	± 0.08
1	1.1– 1.5	1.33	± 0.12
5	6.1– 6.5	6.24	± 0.13
10	12.3–13.1	12.61	± 0.25
20	19.9–21.2	20.31	± 0.35
40	39.5–40.3	39.95	± 0.25
45	46.3–49.1	47.34	± 1.06
50	48.0–50.1	49.18	± 0.49
60	49.0–50.7	49.76	± 0.75
75	49.2–51.2	50.38	± 0.58

NOTE: *Conditions as in Fig. 4.*

values for each level of circulin, as well as the means and standard errors. Fig. 4 and Table II present comparable data for 10 assays based on the release of P^{32}-labelled compounds. In both figures the proportional relationship of release to circulin concentrations is evident.

The above studies were made with cell densities of 10^7 organisms per ml., as determined by direct counts. Under such conditions the range for the assays is from 1 to 45 μgm. per ml. However, as is illustrated by Fig. 5, this range can be shortened or extended by a decrease or increase respectively in the cell density. For instance, when 10^{10} organisms per ml. are used, the range of the assay is from 1 to 100 μgm. per ml.

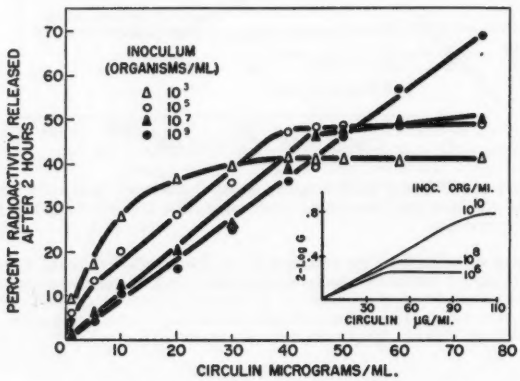

FIG. 5. Per cent of radioactivity and amount of "260 mμ absorbing materials" (inset) released from various concentrations of P^{32}-labelled and normal cells, respectively, of *Escherichia coli* ATCC 26 after two hours of incubation (6°–8° C.) in the presence of various levels of circulin. When the test organism was used in a concentration of 10^{10} cells per ml., instead of 10^7 as used in the other experiments reported in this paper, the useful range of this assay is from 1 to 100 μgm. of circulin per ml.

Accuracy of the Procedures

As a check on the applicability of these procedures for the assay of circulin in fermented broths, known amounts of circulin were added to two fermented media, which then were analyzed for circulin by both methods. Each sample was assayed in triplicate. Table III shows the percentages of circulin recovered when the release of "260 mμ absorbing materials" was used as the basis of the assay, while Table IV presents the recoveries for the assay with

TABLE III

RECOVERY OF ADDED CIRCULIN WHEN RELEASE OF "260 mμ ABSORBING MATERIALS" WAS USED AS BASIS OF ASSAY

		Micrograms circulin added/ml.				
		0	10	100	10	100
Medium	Replicates	Total circulin found			Per cent recovery	
Natural*	1	110	118	200	80	90
	2	106	118	210	120	104
	3	110	118	216	80	106
	Average	109	118	209	93	100
Synthetic†	1	5	16	102	110	97
	2	5.5	15.2	110	97	105
	3	5	14.2	93	92	88
	Average	5.2	15.1	102	100	97

* *Composition in grams per liter: dried brewer's yeast, 20; dextrin, 20;* $(NH_4)_2SO_4$, *10;* KCl, 4; KH_2PO_4, *0.2; and* $CaCO_3$, *16.*

† *Composition in grams per liter: glucose, 10;* K_2HPO_4, *0.5;* KH_2PO_4, *0.5;* $MgSO_4.7H_2O$, *0.2;* $NaCl$, *0.01; leucine, 14; biotin, 0.1 mgm.; thiamine, 50 mgm.; and* $CaCO_3$, *10.*

TABLE IV

RECOVERY OF ADDED CIRCULIN WHEN RELEASE OF RADIOACTIVITY FROM P^{32}-LABELLED CELLS WAS USED AS BASIS OF ASSAY

		Micrograms circulin added/ml.				
		0	10	100	10	100
Medium	Replicates	Total circulin found			Per cent recovery	
Natural*	1	103	112	199	90	96
	2	99	107	199	80	100
	3	109	119	208	100	99
	Average	104	113	202	93	98
Synthetic	1	5.3	15	107	97	113
	2	5	13.9	102	89	97
	3	4.9	15.3	110	104	105
	Average	5.1	14.7	106	97	105

* *See Table III for composition.*

labelled cells. The medium referred to as "natural" is one containing dextrin and yeast extract in addition to several mineral salts (medium No. 7 of Murray *et al.* (9)). The medium termed "synthetic" in its unfermented condition contains only small amounts of organic compounds (1). The average recovery ranged from 93 to 105%.

Discussion

The filter disk – agar diffusion assay for circulin is adequate for routine determinations, but cannot be used with concentrations of less than 10 μgm. per ml. Since release of cellular constituents can be detected when the circulin concentration is as low as 1 μgm. per ml., quantitative determination of this release under standardized conditions offers possibilities for a sensitive and rapid assay. A variety of compounds are released upon damage by circulin, and the method chosen can be adapted to the availability of equipment. The aim of this paper is not so much to offer a fully developed assay method as to illustrate the possibility of this approach for the determination of circulin or other compounds that cause release of protoplasmic components. For example, although cell suspensions containing 10^7 cells per ml. were used in most of the experiments reported here, we have found that the useful range of the assay can be extended to limits from 1 to 100 μgm. per ml. by employing cell densities of 10^{10} per ml. Saline solution or distilled water as suspending medium for the test organism gives assay results that are similar to those obtained when buffer is used. Incubation at higher temperatures (25° C. or 37° C.) rather than at 6° to 8° C. results in very high blank values and therefore was abandoned as an experimental practice. The use of low incubation temperatures is also advantageous in that it minimizes the growth of contaminants.

Inasmuch as the method is based on the determination of protoplasmic constituents, it became important to find out whether the method could be used for the assay of circulin in fermentation broths that might contain a variety of cellular constituents either as ingredients (for example, yeast extract) or as products of growth. Recoveries of added circulin indicate that the interference by such materials was small. However, there are situations in which the liquid to be assayed for circulin might contain so much interfering material that the assay would become inaccurate. For instance, in studies on the mechanism of circulin we have been following the distribution of circulin between cells in very high concentrations (10^{12} per ml.) and the supernatant liquid. Such suspensions release so much "260 mμ absorbing material" that the blank values are too high to permit a satisfactory determination. For such situations the assay based on the release of radioactivity is useful. That is, although the liquid to be analyzed for circulin contains large amounts of phosphorus released by the cells in the experiment mentioned before, the determination of circulin becomes possible when the assay is performed with cells grown on radioactive phosphorus.

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THE PROTEIN CRYSTALS OF *BACILLUS THURINGIENSIS* BERLINER[1]

By C. L. Hannay and P. Fitz-James[2]

Abstract

On sporulation the cells of the insect pathogen *Bacillus thuringiensis* contain at one end a spore and at the other end a diamond shaped crystal. A method has been developed for the separation of the alkali dispersible crystal material from spores, vegetative cell debris, and their alkali soluble components. Two methods are given for the preparation of suspensions of intact crystals free from other material. One method depends on the mechanical disruption of the spores, the other on germination and autolysis of the spore contents. After either method clean suspensions of crystals were obtained by washing and differential centrifugation. The purest preparations have yielded a substance precipitable with trichloracetic acid, possessing the ultraviolet absorption characteristics of protein, containing over 17% nitrogen and at least 17 amino acids, but no phosphorus. Examination of the crystals with the electron microscope has shown that they have a tetragonal form and possess some fine structures; no virus particles were found in alkali dispersed crystal protein.

Introduction

In 1911, Berliner (2) isolated from diseased flour moths (*Ephestia kühniella* Zell.) an insect pathogen which in 1915 (3) he named *Bacillus thuringiensis* and described as a Gram-positive peritrichously flagellated spore-forming rod. He observed that after cessation of vegetative growth, in one half of the bacterial cell a spore was formed, and in the other half the material that was not used in the process of sporulation condensed and formed first a spherical and, later, an irregular rhomboid form which he called a "Restkörper" or remaining body. Mattes in 1927 (13) confirmed Berliner's observations and stated that on staining sporing cultures of *B. thuringiensis* with Giemsa, a bright red spot is often visible in the "sporen anlage" and a similar granule is also to be seen in that part of the cell which later becomes the Restkörper. He speculated that the granules might be part of the bacterial nuclear system. Mattes included in his paper accurate drawings of the Restkörper but did not draw attention to their crystalline shape or suggest that they might be connected with the pathogenicity of the organisms. It was found by Smith, Gordon, and Clarke (18) in 1946 that the Mattes strain of *B. thuringiensis* was culturally and morphologically related to *B. cereus* but differed from *B. cereus* in that it was pathogenic for insects and there was a tendency for the spores to lie obliquely in the cells. Steinhaus has, during the past 10 years, stimulated an interest in insect diseases in general and, in particular, has focussed attention on *Bacillus thuringiensis* Berliner which he found in field trials to be pathogenic for the alfalfa caterpillar (19). In 1953, Hannay (10), while studying sporulation in this organism, rediscovered the "Restkörper" and

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reported that the spores were invariably accompanied by what appeared to be diamond shaped crystals, so that each bacterial cell contained at one end a spore and at the other end a crystal (Fig. 3). A sporulated culture consisted of free crystals, spores, and vegetative debris. Similar pathogens from a number of countries were examined and found to produce similar inclusions. These observations led to speculation that inclusions might be associated with pathogenicity and that crystal formation might be a genetical characteristic of the organism connected with the formation of a toxic substance encouraging septicemia of the insect larvae. By analogy with the insect viral polyhedral bodies the crystals might arise by the action of a virus. Infectivity tests by Steinhaus in 1954 (20) on 51 strains of spore forming bacteria indicated that the strains classified as *B. thuringiensis* were the most virulent. Every strain of *B. thuringiensis* was found to contain inclusions. In the same paper Steinhaus described the isolation, and provided excellent phase contrast pictures, of strains isolated from scale insects which, instead of the characteristic diamond inclusions, possessed rhomboidal to cuboidal inclusions.

Angus (1) working with *Bacillus sotto*, which is the Japanese equivalent of *B. thuringiensis*, found that if old spored cultures were treated with clarified silkworm gut juice (pH 9.5–10) an extract could be obtained which was highly toxic to silkworms. Microscopical examination of the old cultures showed them to be composed of crystals, spores, and vegetative debris. The effect of a water suspension of crystals and spores on silkworm larvae was tested by both injection and feeding. Injected insects died from septicemia; fed insects suffered first from paralysis then septicemia. A similar suspension was treated with dilute alkali until the crystals had dissolved and the spores were removed by centrifugation. The spores and the supernatant were then fed and injected separately and together. The spores freed from the crystal material were non-pathogenic when fed but caused septicemia when injected. The supernatant had no effect when injected but caused paralysis and death when given orally. It was concluded that the evidence was sufficient to indicate that the crystal material might be the cause of the paralysis in the silkworm and, in some manner, be connected with the invasion of the larval tissue by the bacteria. The studies reported here, indeed, indicate that such suspensions do contain some debris from vegetative cells and lysed spores which might itself be toxic.

The present paper discusses work which is exploratory and maps some of the difficulties encountered in connection with: the isolation of the crystal material by alkali fractionation of a mixture containing crystals, spores, and vegetative debris; the preparation of crystal suspensions free from spores; chemical analysis of the crystals and the examination of the crystals with the light and electron microscope. The word crystal is used in preference to paracrystal because neither word has precedence until the character of the body is known.

The fact that an organism as small as a bacterium can isolate material and form such a regular shaped crystal should give pleasure to and evoke the interests of peoples of various disciplines regardless of its effect on the insect.

Chemistry

Chemical Methods

Dry Weights

Aliquots containing 5–15 mgm. of crystal were taken by pipette from a suspension of crystals, dried overnight at 80°, followed by drying to constant weight at 100°–110°. The dried samples, preparatory to weighing, were stored in a vacuum desiccator over P_2O_5.

Phosphorus Determinations

Total phosphorus was determined by the King method (12) with the *p*-methylaminophenol sulphate (metol) reducing agent modification introduced by Gomori (9).

Phosphorus determinations are expressed as a per cent of the dry weight of material examined.

Kjeldahl Nitrogen

Aliquots of a suspension of crystals containing 10–100 μgm. N were digested for one hour in a selenium, copper sulphate, potassium sulphate, sulphuric acid mixture, and were then heated for 15 min. with two drops of 30% hydrogen peroxide solution. Ammonia in the digested sample was distilled from a Markham apparatus into boric acid (6) and titrated with *N*/35 hydrochloric acid using an Agla micropipette. The nitrogen determinations are expressed as a per cent of the dry weight of material examined.

Amino Acid Chromatography

The crystals were hydrolyzed for 8–12 hr. at 110° in sealed glass tubes with 50–200 times the sample weight of constant boiling hydrochloric acid. After hydrolysis the tubes were cooled, opened, and the sample evaporated to dryness at 37° over potassium hydroxide and calcium carbonate in a vacuum desiccator. The amino acids were dissolved in a small volume of 50% aqueous *n*-propanol and aliquots equivalent to 100–200 μgm. of original protein were applied to 11 × 11 in. sheets of Whatman No. 1 paper. The chromatograms were developed in the methanol–water–pyridine and *tert*-butanol – methyl ethyl ketone – water–diethylamine solvents of Redfield (16). In testing for the presence of amino acids other than α-amino acids, aliquots of the hydrolyzate were run on paper that had been sprayed with a 0.05% (w/v) solution of cupric acetate in ethanol and allowed to dry (21). The chromatograms were run with *m*-cresol as the solvent. Tryptophane was detected by hydrolyzing the protein in 5 *N* NaOH at 100° for 18 hr., and though the hydrolysis was incomplete, the hydrolyzate was neutralized, desalted (5), and chromatographed.

Methods for the Preparation of Pure Crystal Suspensions

Preparation of Crystal and Spore Material

The strain of *Bacillus thuringiensis* Berliner used in this work was supplied by Dr. E. Steinhaus and was the same strain used by Hannay (10). The

bacteria for these experiments were grown on Howie and Cruikshank's medium (11) supplemented with 0.5% casamino acids (Difco technical) or on Difco nutrient agar.

The organisms were grown using a method similar to that used by Steinhaus (19). Six-liter Povitsky bottles containing 500 ml. of medium were inoculated with a hand atomizer containing a water suspension of a spored culture. After sporulation and crystal formation was complete a small quantity of distilled water was added to each bottle and the surface growth loosened with a bent glass rod. The suspension was pipetted from the bottles and filtered through a pad consisting of eight layers of cheesecloth. This suspension, now free from lumps of agar, contained crystals, spores, and vegetative debris.

Attempts at the separation of crystals from spores and vegetative debris, or the separation of crystals and spores from vegetative debris by differential centrifugation, failed. During successive water washes of the mixture it was observed that some of the vegetative cells became less dense and could be readily separated from the rest of the mixture. The ultraviolet spectra of the water washes indicated that the purines and pyrimidines in the vegetative debris were being degraded during the washing. To hasten the removal of nucleoprotein derivatives from the vegetative debris the mixture was suspended in molar sodium chloride solution and incubated at 37° for 18-24 hr. The suspension was then centrifuged and the ultraviolet spectrum prepared on the supernatant. The spectrum now resembled that of purine and pyrimidine bases (maximum at 258 mμ). The pellet was given four more washes in saline solution at 37°. The fourth wash failed to remove any nucleic acid material and after four additional water washes most of the vegetative material was easily separated from the crystals and spores by centrifugation.

The centrifuged pellet now consisting mainly of crystals and spores was treated with alkali to disperse the crystal material (10) (Fig. 5), so that the crystal material could be separated from the spores. Chemical analysis of the dispersed material obtained by this method indicated contamination with extraneous matter to be a possibility which served to emphasize the necessity of obtaining a crystal preparation free from spores.

Preparation of Pure Crystal Suspensions

Separation of crystals from spore–crystal mixtures without visible damage to the crystals was achieved by either of two methods, both of which depended upon the destruction of the spores and subsequent separation by differential centrifugation of the lighter spore debris from the heavier crystals.

Method A. Mechanical Disruption of Spores

The disruption of bacterial spores suspended in water is followed by "lysis" of the spore contents (8). The washed crystal–spore mixture was suspended in water containing a small drop of tri-*n*-butyl citrate and No. 14 ballotini beads and shaken in the Mickle disintegrator (14). This procedure permitted the complete separation of crystals from spores. The effect of the method on crystals and spores is illustrated by Figs. 6 and 7; the disrupted spore cases,

including one showing a rent and an undamaged crystal, are shown in Fig. 6; the final pure preparation of refractile crystals is illustrated in Fig. 7.

After growth on Howie and Cruikshank medium, crystals and spores were separated from the vegetative debris by the methods previously described. If the suspension still contained any intact spores the process of disruption and washing was repeated. Because the smashed spore suspension underwent considerable change in a few hours even on storage at 4°, the crystals were separated from the spore debris immediately.

The final crystal suspensions were chalky white, non-flocculent, and could be stored indefinitely in the refrigerator.

Method B. Spontaneous Germination and Autolysis

The spores of *B. thuringiensis* when harvested from Difco Nutrient Agar usually germinated spontaneously and autolyzed. When it occurred, this natural phenomenon was used to prepare pure crystal suspensions. Unfortunately no information is available on the optimum conditions required.

Crystal–spore suspensions were prepared as previously described and stored in the refrigerator as a thick water suspension. After 8–12 days' storage the spores had usually germinated and autolyzed but to ensure maximum autolysis the suspension was then incubated overnight at 37°. The crystals were separated from the spore debris by repeated washing and differential centrifugation.

The Preparation of Crystal Protein by Successive Alkali Extractions of Crystal–Spore Mixtures

When sufficient $N/1$ sodium hydroxide solution was added to a crystal–spore suspension to give a final pH of 12.5, the crystals were completely disintegrated but the spores retained their refractility and viability. This crude alkali extract contained a large amount of material which was precipitable with trichloracetic acid and which had a nitrogen content comparable to that of proteins. No precipitate was obtained from a similar extract of a control preparation of *B. cereus* spores. The washed crystal–spore suspension of *B. thuringiensis* in contrast to the *B. cereus* spore suspension contained some vegetative debris which might have contributed a considerable portion of the total protein obtained by the alkaline extraction.

The high pH required to bring the crystals into solution permitted an alkali fractionation of the alkali extractable proteins present in a washed crystal–spore suspension. The crystal–spore suspension was successively extracted at increasing pH levels, and after each increment of sodium hydroxide solution the morphology of the crystals and spores, the phosphorus and nitrogen content of the extract, and the isoelectric point of any protein present were determined. The results are summarized in Table I.

On the addition of alkali at room temperature to a suspension of crystals, refractility is lost at about pH 10.2 (at higher temperatures, for example, at 30°–37° refractility is lost at pH 9.2–9.5). The first extraction consisted of

TABLE I

THE NITROGEN AND PHOSPHORUS CONTENT OF PROTEINS EXTRACTED FROM DIFFERENT BATCHES OF CRYSTAL–SPORE MIXTURES AT SUCCESSIVE pH'S, AND THE MORPHOLOGY OF THE EXTRACTED CRYSTALS

	Characteristics of proteins in solution			Insoluble residue
Successive extractions at pH	Method of preparing proteins	Nitrogen, % of dry weight	Phosphorus, % of dry weight	Crystal morphology
7–10.0	When neutralized a gel formed which was centrifuged and washed. Variable yields	12.8–13.6	0.08–0.13	Refractile
–10.7	When neutralized no gel formed. Protein precipitated at isoelectric point, pH 4.5–4.6. Small yields	13.3–13.7	0.08–0.19	Loss of refractility
–11.8	Protein precipitated at isoelectric point, pH 4.5–4.6. Small yields	13.3–13.6	0.08–0.19	Swelling to form "ghosts"
–12.5	Protein precipitated at isoelectric point, pH 4.9–5.2. Large yields	14.0–17.1	0.00–0.04	No crystal structure seen

adding alkali until the pH reached 10, at which point the crystals were still refractile. The mixture was centrifuged, the supernatant removed, and its pH adjusted to 7. The solution became viscous and on standing a gel formed which was washed by centrifugation and analyzed. In addition to the gel protein the supernatant contained a lesser amount of a protein which did not sediment during high speed centrifugation at 20,000 *g*.

The pellet from the first extraction was resuspended in water, and alkali was added to bring the pH to 10.7. All the crystals had lost their refractility but while increasing in size they maintained their shape. The suspension was centrifuged and the supernatant analyzed and found to contain a small amount of protein with an isoelectric point at pH 4.5–4.6.

The third extraction was prepared at pH 11.8. The crystals at this point were even more swollen and ghost-like, and could be separated from the heavier spores as a jelly-like mat. After centrifuging, the supernatant contained a small protein fraction similar to that of the second extraction. The swollen crystals were still suspendible in water and in the fourth extraction the pH was raised until they had completely disintegrated (pH 12.2–12.5). A large yield of protein was obtained which had a higher isoelectric point, higher nitrogen and lower phosphorus content than the proteinaceous substances isolated in the first three extractions.

It was at first thought that the various protein fractions might have been derived from the crystals particularly fractions 2 and 3 which were obtained after the crystals had swollen. Later, pure crystals were found to contain only protein similar to that of the last extraction.

Studies on Pure Crystal Preparations

Comparison of the Alkali Extraction of Crystals with the Alkali Extraction of a Crystal–Spore Suspension

In the previous work on the successive alkali extraction of a crystal spore and vegetative cell mixture the source of the different proteins obtained in the alkali fractions was unknown. Now that suspensions of crystals were available it was possible to make the necessary tests.

The curve obtained on plotting pH against ml. of sodium hydroxide added during the "titration" of the crystal spore mixture was not smooth and the irregularities appeared to coincide with the release of the protein fractions. The "titration" was therefore repeated on a suspension of pure crystals. A water suspension of crystals of unknown weight was "titrated" at 25° with sodium hydroxide solution and after each addition the pH was measured with glass electrodes. From the results a smooth curve free from the irregularities noted in the "titration" of the crystal spore mixture was obtained and is illustrated in Fig. 1.

At the same time an approximation to the isoelectric point of the dispersed crystal protein was obtained by back-titrating the solution of the crystals obtained at pH 12.55 with 0.1 *N* hydrochloric acid and measuring the pH and the optical density of the solution at 450 mμ. As the high concentration

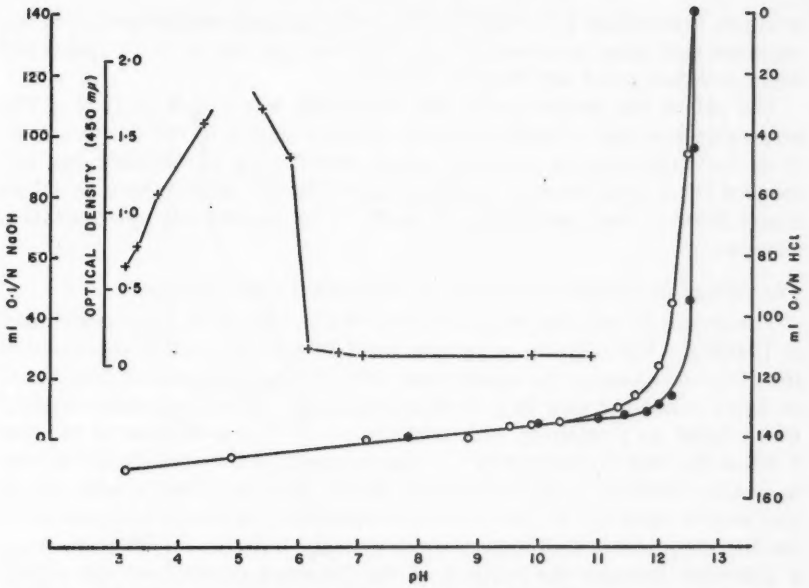


FIG. 1. "Titration" curves of crystal protein (25°). ●—● Titration with 0.1 *N* NaOH (starting at pH 7.8). ○—○ Titration with 0.1 *N* HCl (starting at pH 12.55). x—x Change in optical density of the crystal protein on acid titration.

of protein necessitated a dilution of the solution to a constant volume before measuring the optical density, any dilution effect of the added acid was obviated. The isoelectric point under these conditions lay between pH 4.9–5.2 and was similar to that of the protein in the fourth extraction obtained from the crystal spore mixture. The results were plotted and are illustrated together with those of the previous experiment in Fig. 1.

Finally, suspensions of crystals were extracted at the pH levels used to fractionate the alkali soluble proteins of the crystal spore mixture. A suspension of crystals was added to three test tubes and 0.01 and 0.1 *N* NaOH solution added to give a series of pH levels comparable to those used in the fractionation of the alkali soluble proteins of the crystal spore mixture. The suspensions were then centrifuged at 10,000 *g* and the nitrogen content of the supernatants determined.

When the pH of the first tube was raised to 10 the crystals had lost their refractility and the chalky whiteness of the suspension decreased. After centrifuging the supernatant contained no nitrogen.

The pH of the second tube was raised to 11.8. The crystals were now swollen and ghost-like but were completely precipitated on centrifuging. The supernatant again contained no nitrogen.

As these two alkali extractions at pH 10.0 and 11.8 of pure crystals contained no nitrogen whereas the comparable extractions of crystal spore

mixtures (extractions 1, 2, and 3 in Table I) contained soluble protein it was apparent that these extracted proteins had been released from the spores and vegetative debris and not from the crystals.

The pH of the suspension in the third tube was raised to 12.2. The suspension was now a colorless viscous solution similar to the fourth extract from the crystal–spore mixture. After centrifuging at 10,000 *g* for five minutes the crystal solution yielded a clear jelly-like pellet which would no longer form an even suspension in water. The supernatant now contained nitrogen.

The Ultraviolet Absorption Spectra of Dispersed Crystal Protein

The crystal protein was dispersed in 0.1 *N* alkali at pH 12.2 and centrifuged at 10,000 *g*. The jelly-like pellet contained 84% of the total nitrogen and of the 16% remaining in the supernatant, 89% was precipitated on addition of an equal volume of cold 20% trichloracetic acid. The nitrogenous material which failed to precipitate with trichloracetic acid was equivalent to some 1.8% of the total crystal nitrogen. The treatment of the crystals with sodium hydroxide solution to pH 12.2 might, beside dispersing the protein, split it into smaller units of variable size and complexity. While the homogeneity of the dispersed protein with respect to size would be tested on the ultracentrifuge, a difference between the proteins of the dispersed crystal and the protein remaining in the supernatant after centrifuging at 10,000 *g* could be demonstrated by comparing their ultraviolet absorption spectra. Since the molecular weight of the proteins was unknown and the determination of the exact dry weight of small quantities of proteins suspended in alkali or salt was unsatisfactory, the comparison of the ultraviolet absorption spectra of one protein with that of another protein had to be on some basis other than dry weight or molecular weight. In 1948 Chargaff and Zamenhof (7) introduced the method of expressing the absorption spectra of nucleic acid polymers of unknown complexity as atomic extinction coefficients with respect to phosphorus. It was found convenient when comparing proteins to adopt a similar method and substitute nitrogen for phosphorus in the conversion formula and express the ultraviolet absorption values of the proteins as ϵ (N). The value ϵ (N) is then defined as the atomic extinction coefficient of the protein with respect to nitrogen. ϵ (N) = 14.0 E/cl, where E = extinction or log I^0/I, c = the Kjeldahl nitrogen concentration of the solution in gm./liter, and l = thickness of the cell in cm.

The ultraviolet absorption spectrum of the complete crystal protein dispersed in sodium hydroxide at pH 12.2 was compared with the spectrum of the fraction remaining in the supernatant after centrifuging at 10,000 *g*. The spectra were obtained with a Beckman model DU spectrophotometer and the results are illustrated in Fig. 2. In general a comparison of the absorption spectrum of the fraction with that of the whole dispersed protein shows marked similarities but there are differences in the 280 and 290 mμ region.

The high pH used to disperse all of the protein may have caused a variety of changes in the protein, but until there is a correlation with a biological test

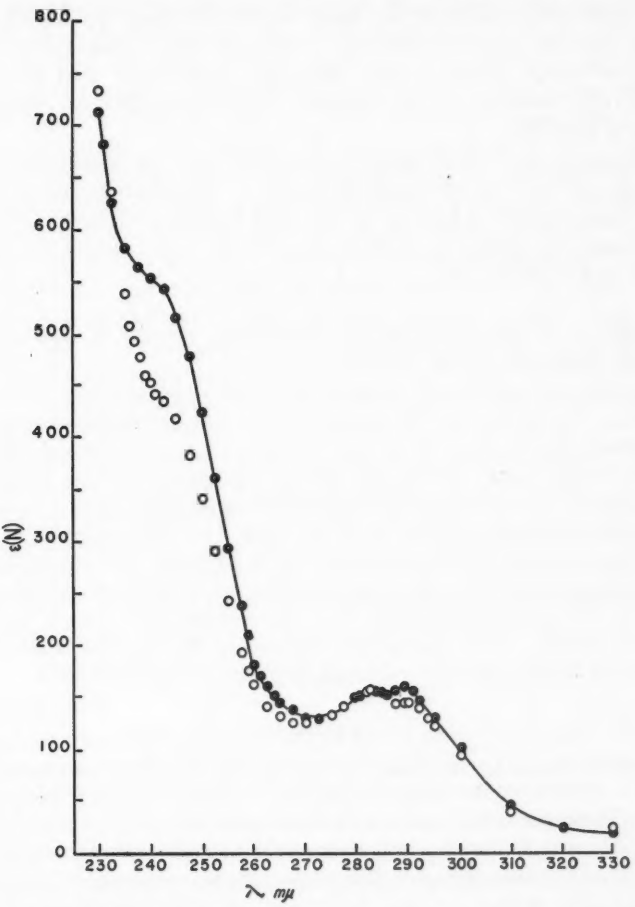


FIG. 2. Ultraviolet absorption curves of crystal protein. ●—● Crystal protein dispersed in sodium hydroxide. ○—○ Fraction remaining in supernatant after centrifuging at 10,000 *g*.

(e.g. toxicity to insect larvae and serological tests), the best method of dispersing the protein with the least damage to any toxic principle will be difficult to assess. Quantitative information on the relative amounts of non-sedimentable protein might provide sufficient evidence as to whether the crystals contain more than one protein.

Amino Acid Chromatography

The amino acids of an acid hydrolyzate of the crystals were chromatographed to determine whether the crystals were composed of few or many amino acids. The examination was superficial and therefore not exhaustive.

Amino acids were found on the spots usually occupied by arginine, lysine, aspartic, glutamic, glycine, histidine, alanine, proline, serine, tyrosine, valine, leucine, isoleucine, phenylalanine, threonine, methionine, and tryptophane. No tests were made for cysteine–cystine. No amino acids other than α-amino acids were detected.

An aliquot of the hydrolyzate was distilled from the Markham apparatus into boric acid and titrated with $N/35$ HCl. The hydrolyzate contained a volatile base equivalent to 11% of the total Kjeldahl nitrogen. If this base were ammonia it seems not unlikely that the protein contains the amides of glutamic and aspartic acid.

A Comparison of the Nitrogen and Phosphorus Content of Batches of Pure Crystals Prepared by Two Methods

A number of batches of crystals were prepared by two methods: *A*, mechanical disruption of the spores in the Mickle disintegrator, and *B*, spontaneous germination and autolysis of the spore contents followed by removal of the spore debris by differential centrifugation. After either method the crystals were washed repeatedly in distilled water. Nitrogen and phosphorus determinations are given in Table II. The variation in nitrogen content from 16.3–17.5% is believed to be mainly due to the variation in the purity of the preparations, but might reflect a real difference in the nitrogen content of the crystals prepared from *B. thuringiensis* grown on different batches of media. The phosphorus content of the crystals while very low is subject to an even greater variation than the nitrogen content.

TABLE II

NITROGEN AND PHOSPHORUS CONTENT OF BATCHES OF PURE CRYSTALS PREPARED FROM WASHED CRYSTAL–SPORE MIXTURES BY EITHER OF TWO METHODS:

METHOD *A*. MECHANICAL DISRUPTION OF SPORES

METHOD *B*. SPONTANEOUS GERMINATION AND AUTOLYSIS OF SPORES

Method of preparation	Comments on preparation	Nitrogen as % dry weight	Phosphorus as % dry weight
B	Contained % spore debris	16.3	0.002
A	Trace of debris	16.7	0.064
A	Pure crystals	17.5	0.000
A	Trace of debris	16.7	0.038

The nitrogen and phosphorus content of the protein obtained from the alkali fractionation of the crystal, spore, and vegetative cell mixture, namely 14–17.0% and 0.00–0.04% respectively, compares favorably with the corresponding analysis of the protein obtained from these crystal preparations.

The possibility that the crystals contained phosphorus before but not after separation from the spores has not been excluded. In both methods of obtaining crystals, the crystals were subjected to enzymes released after the breakdown of the spores. However, even prior to the release of enzymes from the spores they have been in contact with a variety of enzyme actions during their formation in the bacterial cell both at the time of sporulation and, later, after sporulation when the remaining contents of the cell disintegrate. If the crystals had contained entire viable virus particles then by analogy with known bacteriophage systems they would be expected to survive these vicissitudes and the phosphorus remain unchanged and reasonably constant between batches. The speculation of Mattes (13) on the derivation of the "Restkörper" is in this connection of some interest. If the whole DNA–protein complex of a nucleus acted as the *contamination* for the initiation of crystal formation from the protein of the cytoplasm then the crystals should contain some phosphorus which might be susceptible to the action of enzymes found in the cells and spores.

With the exception of the contamination of the crystal preparation with some spore debris containing a variable amount of phosphorus, all of the possibilities presume the ability of enzymes to enter the crystal and act on any compounds present. It is probable that undue emphasis has been placed on the presence of an amount of phosphorus which is less than that usually found in protein preparations but it is nevertheless a point which requires resolving.

Microscopy

Light Microscopy

Throughout this work two staining methods have been used. The first, Dr. C. Robinow's method of air-mounted nigrosin films (17) has been used to follow the formation of crystals and spores within the bacterial cells, cleanliness of spore–crystal mixtures, the effect of alkali on the crystals, and the disintegration of the spores in the Mickle disintegrator, Figs. 3, 4, 5, 6, 7. Because the specimens are mounted in air the magnifications given are only approximate. During the preparation of batches of crystals and spores, films were frequently made and stained with dilute crystal violet: any spores which while still fairly refractile were nevertheless beginning to germinate or autolyze were stained.

It has been found that crystals are sufficiently large that information can be gained with the light microscope from shadowed specimens, Fig. 8. A suspension of crystals was spread on a glass cover slip and shadowed with 2.5 times the thickness of gold used in shadowed preparations for the electron microscope. The shadows are sufficiently clean to be useful, and an indication of the shape of the crystals can also be obtained by direct observation.

Electron Microscopy

All the magnifications given with the illustrations are regarded as approximations. The microscope used was the Philip's EM 100 A.

Crystal Form

The crystals are very regular in shape (Fig. 9) and it is only occasionally that a misformed crystal is encountered. A reconstruction of their shape from the shadows which they cast has shown them to be tetragonal. The first direct observations on the crystal shape were made by Dr. Keith Porter who kindly prepared thin sections from the crystals embedded in methacrylate resin. All the crystal sections examined were four-sided.

In some preparations the crystals have sharp pointed apices, in others they appear to be either truncated or wedge-shaped. Sometimes the electron density is not uniform throughout the width of the wedge so that the ends of the crystals appear forked. As this defect in the crystal structure has been observed with the light microscope in air-mounted nigrosin films prepared directly from the growth on Petri dishes, it must have arisen either during the formation of the crystal in the bacterial cell or later when the crystals lay free on the medium amongst the bacterial debris.

Crystal Distortion

The crystals have proved to be very pliable and subject to distortion but because of their tetragonal form any gross distortion produced as a result of manipulation either before or after they were placed in the electron microscope is either readily apparent or may be made so.

It has been found necessary to examine with as low a beam intensity as is reasonable, for any expansion or contraction of the supporting membrane has a marked tendency to cause distortion of the crystals; and the suspension of crystals should be so dilute that the crystals do not touch one another when placed on the grid, otherwise for some unknown reason, the touching ends frequently splay out and the crystals lose their regular shape.

Systematic distortion of the crystals peculiar to shadowcast specimens has been encountered. The crystals, as might be expected from their octahedral shape, lie on the surface of the film with one pyramid projecting into the air. The raised tips of the shadowed crystals are usually bent away from the shadow source (Fig. 9); those crystals which lie with their longitudinal axis in line with the shadow source appear straight but may, in fact, be bent down. The bending appeared when the preparations were shadowed with gold, palladium, or uranium, but not in unshadowed specimens. Unshadowed crystals subjected to the heat of the tungsten filament remained unbent but if this treatment was followed by shadowing, the crystals were deformed.

Nigrosin air-mounted films (Robinow's technique). Photomicrographs. × 3600

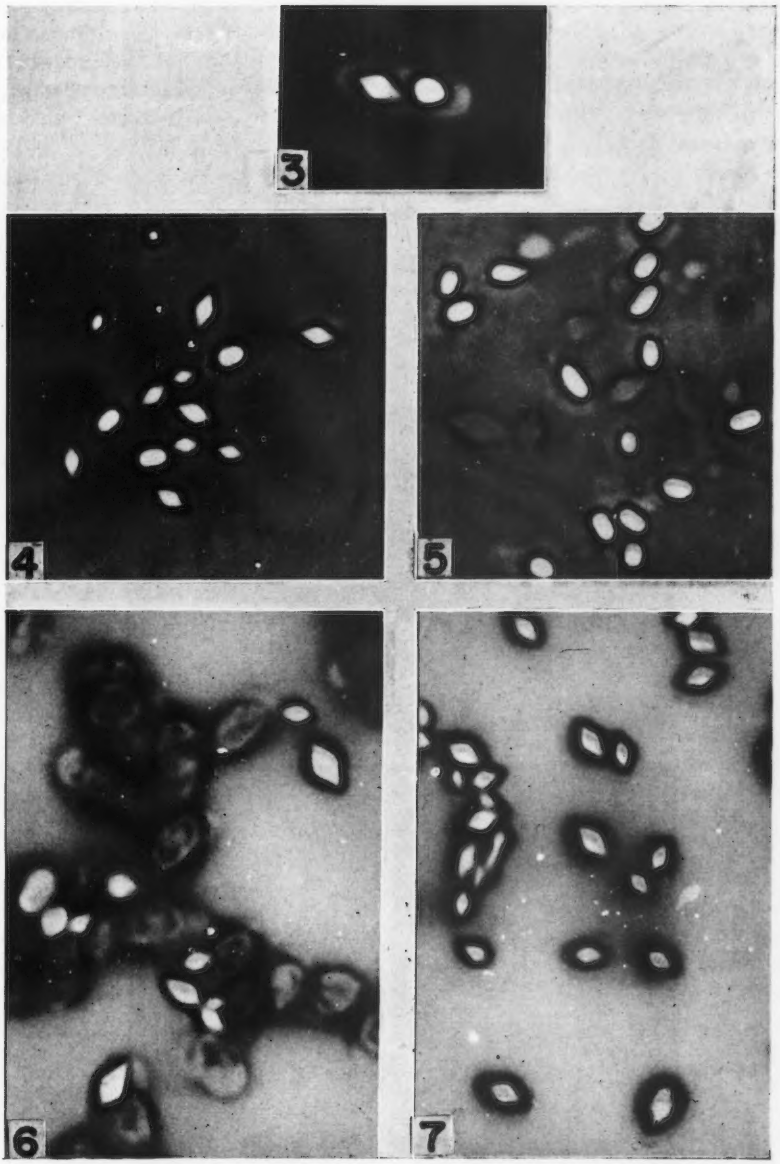
FIG. 3. Refractile crystal and spore lying in a cell in which the other constituents of the cell have lysed.

FIG. 4. Refractile free crystals and spores.

FIG. 5. Non-refractile free crystals and refractile spores after treatment with dilute alkali.

FIG. 6. Refractile crystals and membranes of disrupted spores.

FIG. 7. Pure preparation of refractile crystals after disruption of the spores and removal of the debris.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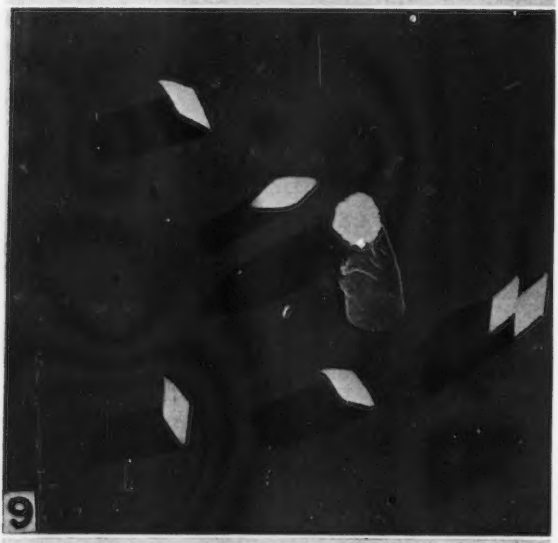
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10

The effect of fixation of the crystals before shadow casting was tested. Drying the preparations over P_2O_5 for 24 hr., fixing in Palade's buffered osmium tetroxide (15) for 15 min. at the different pH's 5, 6, 7, 8, did not reduce the bending; fixation in buffered osmium for two hours or in 0.2% phosphotungstic acid followed by immersion in 1% phosphotungstic acid for one hour lessened the degree of bending but not the number of crystals bent.

It might, at first, be thought that the distortion of the crystals was due to their being bombarded with metal but Dr. T. F. Anderson suggested that the shadowed crystals, when exposed to the electron beam, were acting like bimetallic strips and that if the crystals were shadowed on both sides they would retain their shape. A preparation of crystals was therefore shadowed, turned through 180°, and reshadowed. The majority of the crystals remained straight though a few, which had evidently received less metal on one face than the other, were bent (Fig. 10). The suggestion that the crystals are acting like bimetallic strips carries with it the implication that the side of the crystal that is metal coated expands while the unshadowed side is compressed. An alternative suggestion is that the metal under the influence of bombardment forms a complex with the protein which is more rigid and dissipates more heat than the unshadowed protein. As proteins have a tendency to contract in the electron beam the unshadowed side would contract to a greater extent and the crystals be deformed. Which is the correct explanation has not been determined.

Crystal Structure

In photographic enlargements of shadowed crystals taken at low magnifications the edges of many of the crystals appeared serrated and the surface generally showed a periodicity which was not seen in unshadowed specimens. Variation of the intensity of the electron beam was found to have a pronounced effect on unfixed crystals. If the specimen were placed in the microscope and the beam intensity rapidly increased, the crystals burst producing a lemon-shaped blob on the grid; if the specimen was held in the low intensity beam for a short time, the exterior of the crystal appeared to be "fixed" and, on increase of the intensity, the crystal burst and was surrounded by projecting fingers of material from the split outer layer. The width of these fingers was approximately equal to the distance between successive striae seen on the surface of the crystals. A longer period of bombardment at low intensity sometimes resulted in the fixation of the outer layers followed by a contraction

FIG. 8. A crystal suspension was spread on a glass cover slip, shadowed with gold and photographed with the light microscope. Positive print. × 3600.

FIG. 9. Electron micrograph. Free crystals and a spore. Raised tip of crystals are bent away from shadow source. Negative print. Gold shadowed. × 7500.

FIG. 10. Electron micrograph. Free crystals were shadowed, then the grids turned through 180° and reshadowed. Bending of the raised tips is now confined to a crystal which received an unequal amount of metal on the opposing face. The total amount of gold used was twice that used in the preparation illustrated by Fig. 9. Negative print. × 7500.

of the interior of the crystal when the intensity was increased. In some of these crystals a semblance of structure could be seen (Fig. 11) and the photograph provides a visual interpretation of Bernal and Fankuchen's aphorism, "that crystallinity presupposes an indefinite repetition of identical units in three dimensional space" (4). The serrated edges previously seen on the crystals are now resolved into parallel rows of material which extend round the corners of the crystal. The technique has failed to make it clear whether each pyramid is composed of the same number of rows; whether the pyramids have adjacent basal rows of equal dimensions or one basal row common to both; whether the rows extend throughout the crystal, and whether they form a plate or a spiral about a dislocation in the crystal center. In photographs of a few crystals the rows appear to contain spherical units, but whenever crystals have been intentionally broken in the electron beam the smallest unit has always been the row. This may be an indication that, under these conditions, there is a real difference between the forces holding the rows together and any smaller units which may exist within the rows.

That the treatment of the crystals in the microscope has been drastic is borne out by Fig. 12 which illustrates two crystals with part of their structure removed by the treatment. There is no evidence that the outer layers of the crystal differ in composition from the interior, but it is suggested that the outside, from the time of the crystal's birth in the bacterial cell to the time of its examination in the microscope, has been subject to "denaturation".

The Effect of Alkali on Crystals

If the crystals were analogous to the insect polyhedral bodies then the dispersal of the protein in alkali should reveal the presence of virus particles. In the preliminary work crystals were suspended in 0.02 molar sodium carbonate solution and a drop placed on formvar or collodion-coated grids which, after the preparation had dried, were shadowed and examined in the electron microscope. Many of the ghost forms of crystals contained discrete particles and sometimes the particles were lying free on the supporting membrane. It was, at first, thought that the particles might be viruses but Dr. Keith Porter suggested that they were sodium proteinate artifacts. Crystals were suspended in sodium carbonate solution and the ghost forms spun out and washed by centrifugation in distilled water. Neither these ghost forms or the supernatant contained any particles comparable to those seen in suspensions dried on the grid. The alkali effect was demonstrated by suspending the crystals in 0.02 *M* sodium carbonate solution and placing on the grids a drop of such a size that the whole field could be traversed in the electron microscope. The preparation was air-dried, washed, and shadowed with uranium. In the center of the field where the concentration of alkali had been the highest during the process of drying the crystals contained artifacts (Fig. 13), but at the edge of the preparation where the alkali concentration had been the least there were no artifacts and the crystals appeared to be homogeneous (Fig. 14).

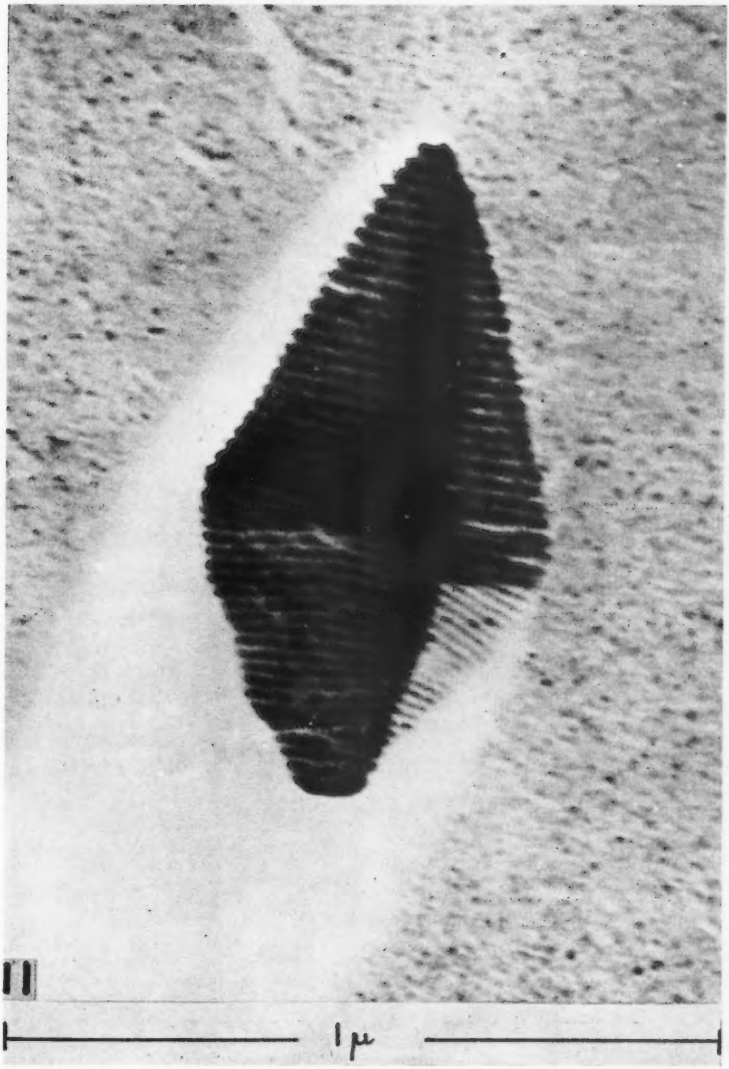


FIG. 11. Shell of protein crystal produced by manipulation of the intensity of electron bombardment. Palladium shadowed. Positive print. × 110,000.

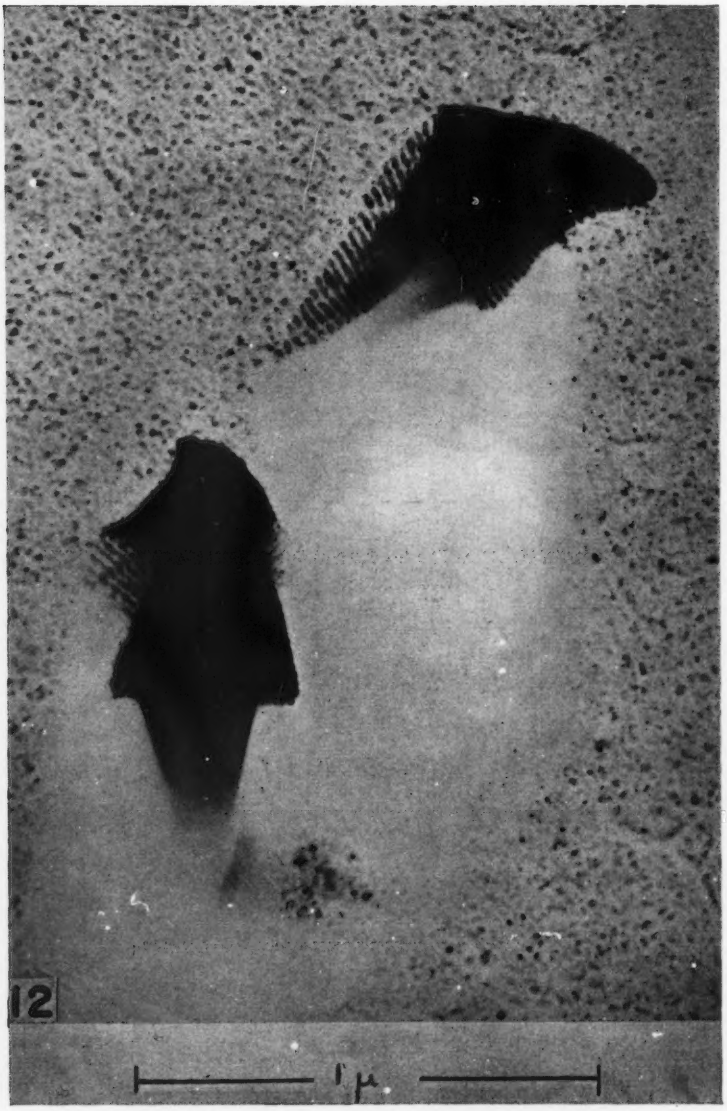
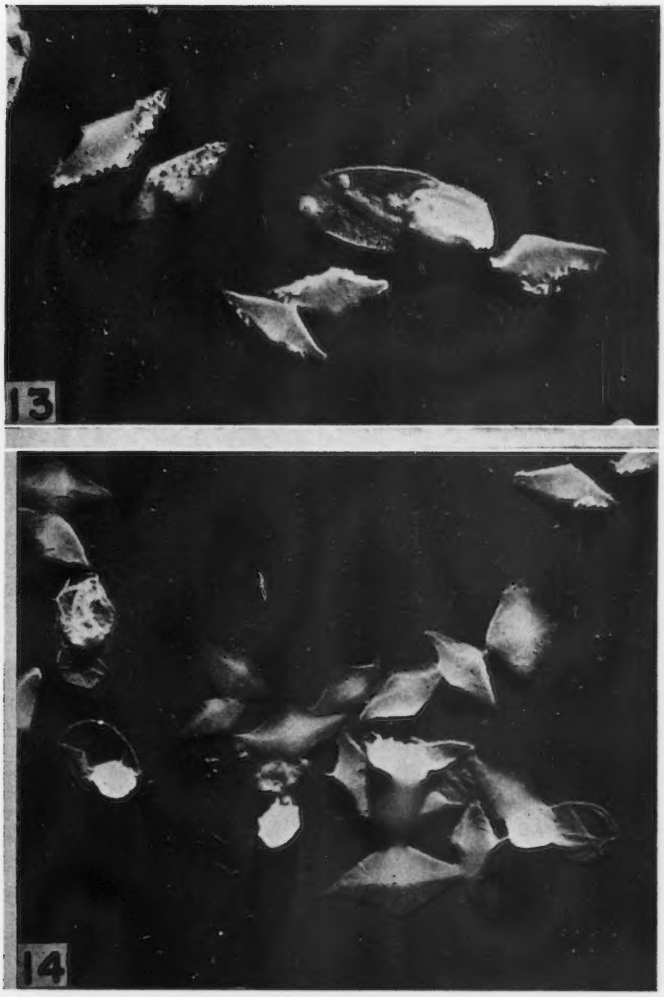


FIG. 12. Two crystals showing the effect produced on their structure by manipulating beam intensity. Palladium shadowed. Positive print. × 71,000.



A drop of a mixture of spores and crystals suspended in 0.02 *M* Na_2CO_3 solution (a concentration which caused the crystals to lose their refractility) was placed on a grid, air-dried, washed in distilled water, shadow cast, and examined in the electron microscope.

FIG. 13. Crystals and a spore in the center of the drop, where the alkalinity increased on drying of the film, contained sodium proteinate artifacts on and within the dispersed crystal protein. Uranium shadowed. Negative print. × 10,000.

FIG. 14. Crystals and spores at the edge of the drop where the least increase of alkalinity occurred shows the dispersed homogenous protein of the crystal (center of the picture). Uranium shadowed. Negative print. × 7500.

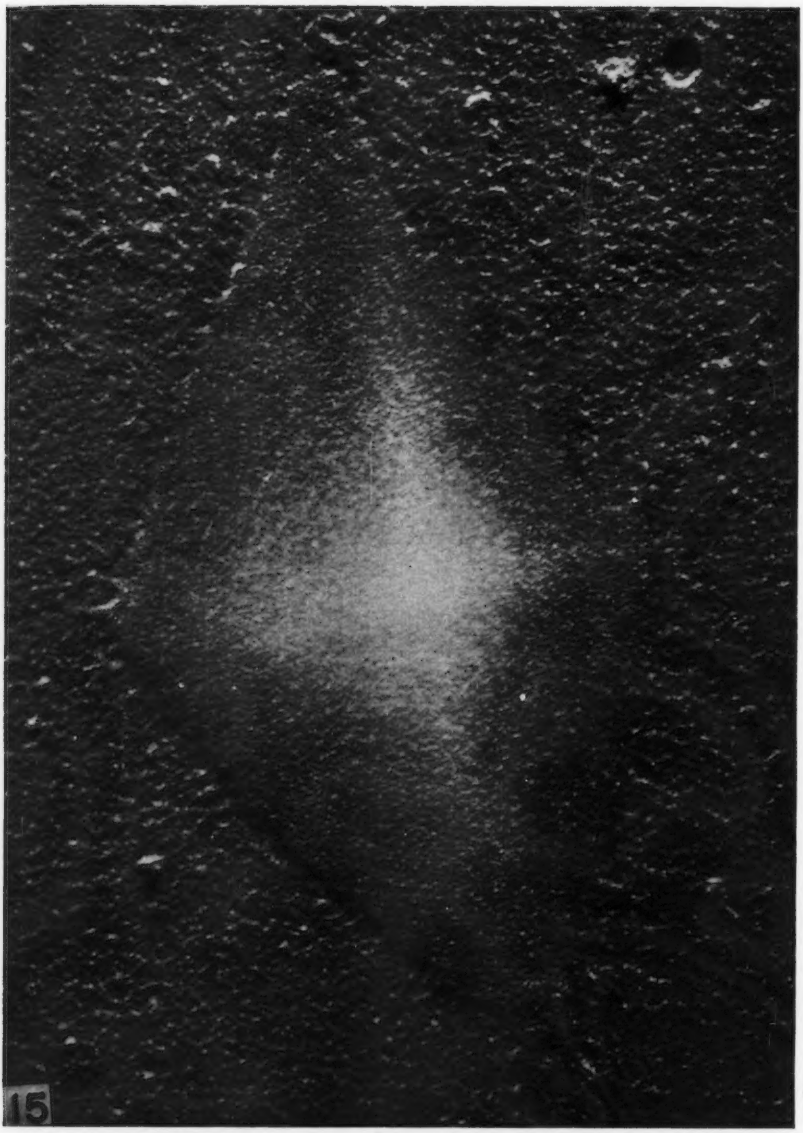


FIG. 15. Alkali treated protein crystal. Negative print. Uranium shadowed. × 66,500.

In the original description of the effect of alkali on the crystals (10) it was stated that after treatment with alkali the crystals disappeared leaving behind a shell or membrane. An examination with the electron microscope failed to reveal any membrane after treatment of the crystals with the alkali. It is apparent that these ghost forms had, with the light microscope (Fig. 5), been mistaken for membranes.

The crystal protein dispersed by alkali appears in Fig. 15 to be made up of small globular units which may or may not be regular in size, but their diameter is less than the width of the rows of material seen in the crystal illustrated in Fig. 12, so that the question of unit size within the rows is unanswered. The unit size of protein obtained on alkali dispersion of the crystal may, while reasonably constant, represent smaller units than that found in the crystalline material. That is, the crystal may not be built of discrete molecules of the size obtained after treatment with alkali but of considerably larger units which are susceptible to mild hydrolysis.

Acknowledgments

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NOTE

THE ANTIGENS CONTAINED IN PREPARATIONS OF SOLUBLE ANTIGEN OF *SALMONELLA TYPHOSA*

By P. J. Moloney and M. J. MacQuarrie

Waters and Moloney (4) reported that a soluble antigen of *Salmonella typhosa* may be prepared by heating a suspension of the bacilli in physiological saline at 56° C. for one hour. The antigen has the property of rendering mice resistant to a challenge dose of typhoid bacilli administered parenterally. It induces in rabbits the formation of antibodies which can be demonstrated both by passive protection conferred on mice and by flocculation in mixtures of antigen and antiserum. Swabb and Reed (3) commenting on the work of Waters and Moloney stated in effect, that Vi antigen was probably not a constituent of the soluble antigen since this latter was derived from a rejuvenated Rawling's strain. This observation of Swabb and Reed is not pertinent, however, because living bacilli of the Rawling's strain used for the preparation of the soluble antigen do possess Vi antigen as shown by agglutination with monovalent Vi serum. On the other hand, the same bacilli after being heated in saline suspension at 56° C. for one hour do not agglutinate with monovalent Vi antiserum. This latter observation might be interpreted mistakenly as indicating that Vi antigen had been destroyed in the process of preparing the soluble antigen.

It was decided therefore to look into the question of what antigen or antigens are present in the soluble antigen preparation described by Waters and Moloney. To this end antiserum to soluble antigen was prepared and examined for antibody content by agglutination and by diffusion technique.

The soluble antigen used for immunization was prepared from a routine lot of concentrated *S. typhosa* vaccine. This latter was made by heating a saline suspension of *S. typhosa* (4.6×10^{10} bacilli per ml.) at 56° C. for one hour after which phenol was added to a concentration of 0.47%. After storage at 4° C. for one month the suspension of killed microorganisms was centrifuged and the clear supernatant separated. Rabbits were inoculated each with 10 ml. of supernatant emulsified in Freund's adjuvant (1). Forty seven days later a second dose of 20 ml. of supernatant without adjuvant was injected into each rabbit. Sixteen days after the second dose, the rabbits were bled and the serum tested for antibodies.

Suspensions of H, O, and Vi antigens and corresponding monovalent sera were obtained from the Central Public Health Laboratory, London, England. Each monovalent serum was found to agglutinate only its corresponding antigen suspension (Table I), hence the antigens were most probably what they were labelled to be.

Results of agglutinations are shown in Table I. It is clear from the results that the antiserum resulting from inoculation of rabbits with soluble antigen contained H, O, and Vi antibodies and hence that the soluble antigen used contained the corresponding antigens.

TABLE I

AGGLUTININ TITERS

Agglutinating sera	Standard suspensions		
	H	O	Vi
Monovalent H	1/80–1/100	0	0
Monovalent O	0	1/320	0
Monovalent Vi	0	0	1/320–1/600
Anti-soluble antigen	1/5000	1/1250–1/2500	1/160–1/320

TABLE II

NUMBER OF ANTIGEN–ANTIBODY ZONES

Anti-sera	Soluble antigens					Culture medium
	1_a	1_b	7	26	30	
Anti Vi	1	1	1	2	1	0
Anti-soluble-antigen	6	4	4	2	6	0

Diffusion analysis of the antigens present in the solution of soluble antigen was carried out according to the technique of Oakley and Fulthorpe (2). The antisera employed were the anti-soluble-antigen serum and the monovalent anti-Vi serum used for determining the agglutinin titers recorded in Table I.

Five preparations of soluble antigen were examined. These were all prepared from routine lots of concentrated *S. typhosa* vaccine in the manner described above. Before the solution of antigen was separated from the killed organisms, the different preparations had been stored at 4° C. for the following number of months: 1, 1, 7, 26, 30. The number assigned to each preparation is the same as the months of storage.

The results of diffusion analysis are shown in Table II. Preparation 1_a was the soluble antigen which had been used in the preparation of anti-soluble-antigen serum and it possessed the typhosa antigens H, O, and Vi (Table I). By diffusion technique this preparation contained at least six antigens, that is at least three antigens in addition to H and O and Vi. Three of the remaining five preparations contained fewer than six antigens. No explanation is

suggested for differences in the antigen content of the preparations. The anti-Vi serum contained at least two antibodies as shown by two zones of precipitation with preparation No. 26.

We wish to thank Dr. M. H. Brown and Dr. Helen Plummer for preparations of typhoid vaccine, and the J. P. Bickell Foundation for a grant.

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